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(54) Title: METHOD FOR EXPANDING PRIMATE B CELLS SELECTIVELY IN IMMUNOCOMPROMISED MICE AND PRODUCING LARGE NUMBERS OF ANTIGEN-SPECIFIC B LYMPHOCYTES FOR THE PRODUCTION OF PRIMATE MONOCLONAL ANTIBODIES

(57) Abstract

The present invention relates to a method for reconstituting immunocompromised mice with primate cells, consisting of the following steps: (a) preparation of an immunocompromised mouse by administration of an antibody against the beta chain of the mouse interleukin 2 (IL-2) receptor, more particularly the monoclonal mouse TM-β1 monoclonal antibody and/or gamma irradiation, (b) intraspleen injection of primate cells into the spleen of the pre-treated immunocompromised mouse of step (a), where these primate cells preferably originate from a primate donor and where this primate donor may have been immunised with a well-defined antigen. This method was found to be highly suitable for expanding primate B lymphocytes in an animal model in a rapid, as yet unequalled manner. Other embodiments of this invention relate to immunocompromised mice obtained by the aforesaid method and also the use thereof as animal models, primate B cell culture produced and primate monoclonal antibodies derived therefrom.

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METHOD FOR EXPANDING PRIMATE B CELLS SELECTIVELY IN IMMUNOCOMPROMISED MICE AND PRODUCING LARGE NUMBERS OF ANTIGEN-SPECIFIC B LYMPHOCYTES FOR THE PRODUCTION OF PRIMATE MONOCLONAL ANTIBODIES

The present invention relates to a method for selectively expanding primate B cells, more particularly primate B lymphocytes, and producing large numbers of antigen-specific B lymphocytes in immunocompromised mice for the production of primate monoclonal antibodies. The object of the present invention is a method for selectively enriching primate B lymphocytes, more preferentially human B lymphocytes, and allowing them to survive and function in an immunocompromised mouse and more preferentially a *scid/scid* (SCID) or NOD/LtSz-*scid/scid* (NOD-SCID) mouse, with the aim:

- in a non-antigen-specific way of:
  - studying the biology of primate B lymphocytes, more preferentially human B lymphocytes.
  - allowing primate B lymphocytes and more preferentially human B lymphocytes to live (survive) in the mouse as a substrate/target for (viral or other) infections which use the B lymphocyte as host cell.
- in an antigen-specific way of:
  - producing primate monoclonal antibodies, more preferentially human monoclonal antibodies in view of the high enrichment of antigen-specific B lymphocytes which are in a suitable state for use as a fusion partner for (hetero)myeloma cells.
  - its area of application is enormous and relates to the production of all conceivable primate or human monoclonal antibodies which can be used for:
    - therapeutic purposes
    - diagnostic purposes
    - in vitro explorative purposes
  - creating gene banks for antigen-specific immunoglobulin mRNAs.

'Severe combined immunodeficient' (SCID) mice are derived from the C.B.-17 strain and have a defective DNA-dependent protein kinase which is essential in the VDJ rearrangement of the antigen receptors. The consequence is that *scid/scid* (SCID) mice cannot produce any antibodies and T cell receptors and thus also do not have any functional B and T lymphocytes. As a result they are immunodeficient (Schuler et al., 1986). SCID mice do have a functional innate immune system (monocytes, macrophages, PMN and NK

cells), as a result of which they are not completely immuno-incompetent and can survive and reproduce in a pathogen-free environment.

Because SCID mice are immunodeficient, they are receptive to xenografts. SCID mice can thus be reconstituted with human peripheral blood leukocytes (HuPBL) or with human foetal tissues, resulting in HuPBL-SCID and SCID-Hu mice, respectively. A phenomenon which occurs to a certain degree is the host versus graft reaction. This is the rejection of the graft by the host's innate immune system. Graft versus host reactions, in which the immune cells of the graft react against the tissues of the mouse, can also be observed.

McCune et al. (1988) showed that SCID mice can be transplanted with human foetal lymphoid tissues and cells. This gives rise to mouse-human haematolymphoid chimeras, which are called SCID-Hu. They found that transplantation of the mouse with human foetal liver cells and thymus tissue resulted in thymocyte growth within the human thymus of the SCID-Hu mouse, without any graft versus host disease occurring. From 3-4 weeks to 10-12 weeks after the injection of the human foetal liver cells, human cells with T cell markers are also transient detected in the peripheral blood of the SCID mouse. However, no human cells are recovered in the murine thymus and lymph nodes, and very few cells are found in the SCID spleen. No human B cells were seen in the SCID mice which were transplanted with human thymus and foetal liver cells, although human Igs occurred in their blood. Specific human immune responses in the SCID-Hu mouse have not yet been published in the literature. The inability of human thymocytes to repopulate the murine lymphoid organs is an important limitation for generating a functional, recirculating human lymphoid system in the SCID mouse. This failure can be explained by the destruction of the circulating human T lymphocytes by SCID myeloid and/or NK cells.

In 1988 Mosier et al. found that the intraperitoneal injection of the HuPBL into SCID mice can result in a stable and long-lasting reconstitution with a functional human immune system in which a secondary immune response can be evoked. The majority of the human cells in the HuPBL-SCID are recovered in the lymphoid organs and in the blood. Two to three weeks after the reconstitution of the SCID, 80-92% of all cells in the spleen were found to be human lymphoid cells. Subsequently, however, Mosier et al. (1989) attributed this high percentage to aspecific staining and stated that the actual percentage of the HuPBL in the spleen of the SCID was much lower.

Moreover, various authors find very few or no human T cells in the lymphoid organs of the SCID during the early post-transplantation period (Abedi et al., 1992; Murphy et al., 1992). Tary-Lehmann et al. (1992) detected no human cells in the periphery during the first 20 days after the transplantation.

Because of the low percentage of human cells in the peritoneal cavity and the lack of migration to other organs, we can conclude that most human cells die off after intraperitoneal injection.

The cells which survive after 20 days migrate to other organs. The human lymphocytes are located predominantly in the lungs, liver or spleen of the chimeric animals. Few cells get into the thymus, bone marrow or lymph nodes.

The considerable loss of the injected human cells in the first few days after transplantation is mainly due to the mouse Natural Killer (NK) cells and macrophages. These cells represent the innate immune system and still show high activity in the SCID mouse (Dorshkind et al., 1985). Elimination of one or both types of cells in the SCID mouse can lead to an improved reconstitution.

Murphy et al. (1992) proved that the murine NK cells can have a negative effect on the transplantation of the human lymphocytes in the SCID. Depletion of the NK population with 20 µl anti-asialo GM1 antibodies led to a better reconstitution. The polyclonal anti-asialo GM1 antibodies recognise the murine NK cells and deplete them after intraperitoneal injection (Habu et al., 1981). The anti-asialo GM1 antibodies remain active for 5 to 7 days. Shpitz et al. (1994) therefore treated the SCID mice with 25 µl of these antibodies one day before the reconstitution and every 5-7 days thereafter. Sandhu et al. (1994) only administered anti-asialo GM1 antibody once and used a lower dose (20 µl instead of 25 µl).

In comparison with 'wild-type' C.B.-17 mice, SCID mice show an increased sensitivity to ionising radiation (Fulop et al., 1986, 1990). In 1997 Kobayashi et al. showed that both the bone marrow cells and the fibroblast cells of SCID homozygotes (scid/scid) were much more sensitive to irradiation than the cells of heterozygous SCID mice, which in turn were more sensitive than ordinary C.B.-17 mice. For bone marrow cells this was the case both in vivo and in vitro. No difference in sensitivity to UV light and chemicals can be detected, however.

Abedi et al. (1992) determined that when the SCID mouse is irradiated before the administration of human PBL this is associated with a more rapid production of human IgG antibodies. In the spleen of a non-irradiated SCID mouse less than 5% human CD3+ (T) cells on average are found 6 to 32 days after the injection of human PBL.

If the SCID mouse is previously irradiated with a dose of 300 rad, the percentage of human T cells in the spleen 12-14 days after reconstitution will be 16% (Shpitz et al., 1994). Radiation reduces the activity of NK and macrophage precursors, and through cell death irradiation also induces a sort of

inflammatory reaction that leads to the production of murine cytokines which, in the event of cross-reactivity, the transplanted human cells can modulate positively. In addition, irradiation frees space in the SCID mouse, as a result of which the injected cells have adequate room for expansion.

Shuler et al. (1986) showed that radiation of 300 rad is more than sufficient to eliminate the activity of the murine NK cells.

Shpitz et al. (1994) proved that irradiation of the mice with 300 rad, combined with the treatment with anti-asialo GM1, markedly improves the transplantation of the human B, T and NK cells. This was apparent from the presence of higher percentages of human lymphoid cells in the spleen.

Gallinger et al. (US-A-5,663,481) have already described a method for the intraperitoneal reconstruction, with human peripheral blood leukocytes, of SCID mice that have been previously treated with an anti-asialo GM1 antibody and radiation.

Another method for selectively reducing the Natural Killer population in the SCID mouse consists of treating the mouse with antibodies directed against the β chain of the mouse IL-2 receptor. The IL-2 receptor β chain is of course part of the IL-2, IL-7 and IL-15 receptor. These interleukins are important growth factors for the maturation and differentiation of NK cells (Biron et al., 1990).

A single intraperitoneal injection of SCID mice with 1 mg anti-IL-2Rβ antibodies (TM-β1) causes a prolonged selective elimination of NK cells. No significant quantities of NK cells can in fact be detected in the spleen up to five weeks after treatment (Tanaka et al., 1993).

The antibodies were produced by immunising a rat with a TART-1 cell line which expresses large quantities of the IL-2 receptor β chain (TART-mβ). The specific, monoclonal rat antibodies were called TM-β1 (Tanaka et al., 1991). The treatment of SCID mice with TM-β1 for the study of leukaemia (Kondo et al., 1993), metastasis (Yano et al., 1996) and liver transplantation (Furukawa et al., 1995) has already been reported in the literature. The use of TM-β1 in the HuPBL-SCID model has not yet been evaluated.

Backcrossing of SCID onto the NOD/Lt strain resulted in the non obese diabetic (NOD)-SCID mouse, which has next to a mature T- and B-cell deficit also a reduced NK activity, macrophage function and serum haemolytic complement activity (Shultz et al, 1995). These NOD-SCID mice are better hosts for the Hu-PBL grafts with a concomitant higher human Ig production when compared to SCID mice (Greiner et al, 1995).

One of the aims of the present invention is to develop a more efficient method for expanding primate cells and more preferentially human cells in an animal model, more particularly in an immunocompromised mouse.

Another aim of the present invention is to develop such a method for expanding primate lymphocytes, more preferentially human lymphocytes in SCID or NOD-SCID mice.

Yet another aim of the present invention is to develop an animal model in which primate cells, more particularly primate B cells, can expand.

Yet another aim of the present invention is to develop an animal model in which human cells, more particularly human B cells, can expand.

Yet another aim of the present invention is to develop a primate and more preferentially a human monoclonal B cell culture.

Yet another aim of the present invention is to develop primate monoclonal antibodies and more preferentially human monoclonal antibodies.

All these objectives have been met by the embodiments of this invention as explained below.

The present invention relates to a method for reconstituting immunocompromised mice with primate cells characterised by intra-spleen injection of primate cells in an immunocompromised mouse.

The present invention relates more particularly to a method as described above wherein the immunocompromised mice are depleted of functional T and B lymphocytes.

The terms "immunocompromised" and "immunodeficient" are sometimes used interchangeably. Immunocompromised or immunodeficient mice refers to mice which are not able to evoke an effective immune response against the xenogenous haemopoietic cells of the primate and more preferentially of the human immune system.

The present invention relates more particularly to a method for reconstituting immunocompromised mice with primate cells as described above wherein said mice are *scid/scid* (SCID) or NOD/LtSz-*scid/scid* (NOD-SCID) mice.

Scid/scid (SCID) mice homozygous for the severe combined immunodeficiency lack functional T and B lymphocytes and are permissive for xenogenous immune cell engraftment (Bosma et al, 1983). NOD/LtSz-*scid/scid* (NOD-SCID) mice lack functional T and B cells and have accompanying defects in nonadaptive immunological function, particularly in natural killer (NK) cell activity, inherent to the NOD/Lt strain background (Shultz et al, 1995). As can be deduced from the examples, NOD-SCID mice supported higher levels of

engraftment of intraspleen transplanted human peripheral blood leukocytes than did SCID mice.

Other immunocompromised test animals (mice and other animal species) which are not able to evoke an effective immune response against the xenogenous haemopoietic cells of the primate and more preferentially of the human immune system can also be used instead of SCID or NOD-SCID mice. Amongs them, the SCID mutant mouse strains such as SCID-Bg (scid/scid-beige/beige) or the Bg-nu-xid (beige/beige-nude/nude-X-linked immunodeficient) mice can be mentioned as an example.

The present invention relates more preferably to a method described above, wherein the immunocompromised mice are pre-treated by administration of an antibody directed to the mice natural killer cells and/or gamma irradiation. Said antibody can be a monoclonal or polyclonal antibody.

The present invention relates more preferably to a method described above, wherein said antibody is directed against the beta chain of the mouse interleukin 2 (IL-2) receptor. Blockade of this receptor in vivo selectively eliminates endogenous mouse NK cell activity in various mouse strains and severely enhance leukocyte survival in the reconstituted immunocompromised mice.

The present invention relates more preferentially to a method described above consisting of the following steps:

(a) pre-treatment or preparation of an immunocompromised mouse by administration of an antibody against the beta chain of the mouse interleukin 2 (IL-2) receptor and/or gamma irradiation,

(b) intra-spleen injection of primate cells into a pre-treated immunocompromised mouse from step (a).

The injection of the primate cells into the pretreated immunocompromised mouse of step (b) can also be intraperitoneal (IP). Injection into the spleen, however, provides a more efficient animal model, because the B cell enrichment is greater.

The present invention further relates to a method as described above wherein the antibody against the beta chain of the mouse interleukin 2 (IL-2) receptor is a monoclonal antibody.

The present invention relates preferably to a method as described above wherein between 50 μg and 5 mg, preferentially between 100 μg and 2 mg, more preferentially between 500 μg and 1 mg, and preferably 1 mg of monoclonal antibody against the beta chain of the mouse interleukin 2 (IL-2) receptor is injected in step (a).

The present invention relates preferably to a method as described above wherein the monoclonal antibody against the beta chain of the mouse interleukin 2 (IL-2) receptor is the TM-β1 monoclonal antibody. The TM-β1 monoclonal antibody was described by Tanaka et al., 1991 and Tanaka et al., 1993. As can be deduced from the examples, the administration of TM-β1 monoclonal antibody prior to the IP or IS transplantation of human cells into the immunocompromised mouse significantly improves the survival, the distribution and the functionality of the xenografts.

Consequently, pre-treatment with an other NK depleting antibody (monoclonal or polyclonal antibody) may possibly also give the same result. Otherwise, new mouse strains, all characterised by additional immune defects (preferably characterised by a more reduced NK activity) next to T- and B-cell deficiency can provide ameliorated *in vivo* incubation systems for primate immune grafts. As a consequence, the man skilled in the art can assume that, when using those animals in the above mentioned method, the pre-treatment of the immunocompromised mice by administration of an antibody directed to the mice natural killer cells probably can be eliminated.

A preferential method according to the present invention concerns a method as described above wherein in step (a) there is also γ radiation between 100 and 900 rad, preferably between 200 and 600 rad and more preferably at 300 rad, 24 hours before transplantation.

The term "primate" refers to any mammal of the order Primates, including man, apes, monkeys, lemurs, and living related forms that are thought to be derived from generalised arboreal ancestors. Whenever, in the present application the term "primate" is used, it preferentially refers to humans or apes. Among the apes, the chimpanzees are preferred.

According to another embodiment the present invention also relates to a method as described above wherein the primate donor is a human.

The present invention further relates to a method as described above wherein the primate cells injected are human cells.

The present invention further relates to a method as described above wherein the primate cells injected are human peripheral blood leukocytes (Hu-PBLs).

This method, of course, does not allow only primate peripheral blood leukocytes or peripheral blood mononuclear cells (PBMC) to be injected into the spleen of the test animal, but also all other cells and cell suspensions, such as primate spleen cells, bone marrow cells, tonsillar cell suspension, cells produced from lymph glands, isolated stem cells ($CD34^-$ or others), and even hepatocytes.

The terms PBL and PBMC are sometimes used interchangeably. PBMC includes lymphocytes, monocytes and NK cells. The terms reconstitution, transplantation and engraftment are used interchangeably.

The present invention further relates to a method as described above wherein the number of human peripheral blood lymphocytes (Hu-PBLs) injected is between 1×10^7 and 1×10^8, preferentially between 1×10^7 and 5×10^7, more preferentially between 1×10^7 and 3×10^7, and preferably 2×10^7.

The present invention further relates to a method as described above wherein the primate cells originate from a primate donor who has been immunised with a well-defined antigen, infectious agent or parts thereof.

The term "well-defined antigen" relates to any self or non-self antigen capable of inducing an immune response in the primate donor which leads to the presence of antigen-specific B cells in the hemopoietic and lymphomonocytic tissues.

The term "infectious agent" refers to any agent (viral or other) capable of causing an infection.

According to another embodiment the present invention relates to a method as described above wherein the primate cells injected includes immune cells and more preferentially antigen-specific B-cells.

The term "immune cells" refers to any cell involved in the immunity of an individual.

The key feature in the present invention is the choice of injection of the primate cells into the spleen. The major advantages of this transplantation route is the expansion of the primate B cell population which has never been documented upon intra-peritoneal engraftment of primate peripheral blood leukocytes and the higher secondary immune response that is induced in comparison with intra-peritoneal reconstituted immunocompromised mice. Injection into the spleen provides an even more efficient animal model because the B cell enrichment is greater. The method of the invention leads surprisingly to a considerable expansion of the B lymphocytes in the early phase of reconstitution.

The present invention concerns a method for expanding primate B lymphocytes and more preferably a method for the activation, proliferation and differentiation of primate B lymphocytes in an animal model in a rapid, as yet unequalled manner.

The number of cells is evaluated with a FACScan (Becton Dickinson), for example.

The method as given in the examples of this invention is characterised by the fact that the immunocompromised mice show at least 80% reconstitution

of the functional human lymphocytes in the spleen, as can be seen in the examples. After 6 days the total quantity of human leukocytes is 82.9% of the total number of leukocytes.

The most important differences between the method of the invention and the method of Gallinger et al. (US-A-5,663,481) are to be found in Tables 3 and 4 of the examples and the discussion thereof.

According to another embodiment the present invention also concerns an immunocompromised mouse reconstituted with primate cells by the method as described above.

In a preferred embodiment, the present invention also concerns an immunocompromised mouse reconstituted with human cells, preferentially human PBLs, according to the method as described above.

The present invention also concerns an immunocompromised mouse reconstituted with primate PBLs, preferably according to the method described above, which is characterised by the fact that 6 to 7 days after the reconstitution more than 80% of the spleen cells are primate cells.

The present invention further concerns an immunocompromised mouse reconstituted with primate cells, preferably according to the method described above, which is characterised by the fact that 6 to 7 days after the reconstitution up to 70 to 80% of the primate cells found in the spleen are CD19+ B cells.

The present invention more preferably concerns an immunocompromised mouse reconstituted with HuPBLs, preferably according to the method described above, which is characterised by the fact that 6 to 7 days after the reconstitution up to 70 to 80% of the human cells found in the spleen are CD19+ B cells.

After intraperitoneal injection of human leukocytes in control or conditioned SCID or NOD-SCID mice, T-cells always constitute the majority of the human cell population present in the peritoneum or the lymphoid organs whilst B-cells only occur in low numbers (Shpitz et al, 1994; Hoffmann Fezer et al, 1992; Hesselton et al, 1995). Using the method of the present invention, 7 days after intrasplenic inoculation, B-cells strikingly predominated in the human cell population (presented in the examples of the invention). B-cell predominance was independent of the mouse strain used or the pre-condition regimen of the animal host. Highest absolute numbers of human B lymphocytes were present in the spleen of TM-β1-treated and irradiated SCID or NOD-SCID mice. Human monocytes and NK cells, originally present in the inoculum, were no more detectable and human T-cells constituted only a minor fraction. The immune responsiveness of the engrafted human B-cells was evidenced by early

and high spontaneous production of circulating human immunoglobulin (Ig) G and IgM (see examples).

According to another embodiment the present invention also concerns an immunocompromised mouse as described above for use as an animal model system.

These reconstituted immunocompromised mice can be used for evaluation of the effect of drugs on the primate and more preferentially the human immune system, for the development of therapeutic agents and immunisation compositions, for vaccine design and development. Such models can also be used for the testing of prevention, treatment, suppression and enhancement of the primate, and more preferentially the human immune response and for the treatment of diseases such as cancer or pathogenic infection.

Intra-splenic injection of primate PBLs into an immunocompromised mouse leads surprisingly to a considerable expansion of the B lymphocytes in the early phase of reconstitution.

The present invention also relates to the use of a reconstituted immunocompromised mouse as described above for the study of the immunobiology of primate hemopoietic and lymphomonocytic tissues.

As can be deduced from the examples, human B lymphocytes become activated, grew vigorously and differentiated into plasmacytoid cells during reconstitution. This process is T-cell dependent. *In vivo* exposition to a recall antigen after cell transfer expands antigen-specific B cell clones prior to fusion resulting in the production of antigen-binding human hybridoma antibodies. Thus, intrasplenic transplantation of primate, more preferably human lymphoid cells in immunodeficient mice provides a model for the study of primate, more preferably, human T cell-dependent B cell activation. This includes B cell Ig class switch and/or affinity maturation (hypermutation), discovery of new T/B-cell co-stimulatory/adhesion molecules, discovery of new B-cell differentiation markers, study on B-cell membrane signalling and intracellular pathways.

According to another embodiment the present invention also relates to the use of a reconstituted immunocompromised mouse as described above for the study of human or animal viruses and more preferentially B lymphotropic human or animal viruses. This application can involve the infection of primate lymphoid cells with lymphotropic pathogens. Since the method of the present invention leads to a considerable expansion of the B lymphocytes in the early phase of reconstitution, said reconstituted immunocompromised mice can be

used as a model to study preferentially B lymphotropic viruses like EBV, HCV and others.

The present invention further relates to the use of a reconstituted immunocompromised mouse, as described above, to analyse the role and function of donor B cells in the xenogeneic graft versus host disease.

According to another embodiment the present invention relates to primate monoclonal B cell cultures produced by isolating the B cells of a reconstituted immunocompromised mouse, described above, and then immortalising these B cells by fusion with a heteromyeloma. Said isolated B cells can also be immortalised by other methods known in the art, for example by EBV infection or genetic transformation.

In a more preferred embodiment the present invention relates to human monoclonal B cell cultures produced by isolating the B cells of a reconstituted immunocompromised mouse, described above, and then fusing these B cells with a heteromyeloma.

According to a specific embodiment, the present invention relates to primate monoclonal antibodies produced by a primate monoclonal B cell culture as described above.

The present invention further relates to human monoclonal antibodies produced by a human monoclonal B cell culture as described above.

The present invention provides a powerful tool for the generation, production, use and exploitation of primate monoclonal antibodies and more preferentially human monoclonal antibodies towards an unlimited series of antigens (including self and non-self antigens). Human monoclonal antibodies have, for example, therapeutic potential against infectious diseases and cancer (Maloney et al, 1994; Crowe at al, 1994). Their production has been limited as ethical constraints prevent the reliable isolation of antigen-specific activated B cells by *in vivo* immunisation. Immunocompromised mice, intraperitoneally transplanted with human peripheral blood leukocytes (Hu-PBL), allow the *in vivo* stimulation of human antibody responses without the usual constraints (Bosma et al, 1983 and Mosier et al, 1988). Human B cells however only represent a minor fraction of the surviving graft, are scattered all over the animal body and thus hard to isolate for subsequent immortalisation procedures. Monoclonal antibodies to recall antigens have been generated from cells isolated from intraperitoneally reconstituted Hu-PBL-SCID through combinatorial gene library generation (Duchosal et al, 1992). This technique remains however highly work intensive in that a high number of clones have to be tested and results in antibodies that are cloned as Fab fragments whose biological activity is inferior to that of complete Ig proteins. Other investigators have established cloned cell

lines from visible Epstein Barr virus-transformed tumours, growing in the Hu-PBL-SCID, that produce antibodies specific for the immunising antigen (Carlsson et al, 1992). These tumours however express only monoclonal or oligoclonal B-cell repertoires (Saxon et al 1991) and the cell lines have rather low antibody production capacity and are generally unstable. In the present invention, immunocompromised mice were engrafted with Hu-PBL directly into the spleen, instead of intraperitoneally, after depletion of the endogenous murine Natural Killer (NK) cell activity by treatment with the anti-mouse interleukin (IL) 2 receptor beta chain Ab. One week after inoculation, human B cells were highly numbered at the site of injection and could easily be recovered, as presented in the examples. The intrasplenic reconstituted immunocompromised mice of the present invention and more preferentially the intrasplenic primate-PBL-SCID or primate-PBL-NOD-SCID model of the present invention allows to easily tap the rich memory compartment of primate antibodies leading to a large repertoire of stable, highly productive hybridomas secreting primate monoclonal antibodies useful in immunohistology and passive immunotherapy.

According to another embodiment, the present invention also relates to a method, as described above, for producing activated and differentiated primate B cells characterised by the following:

- reconstitution of immunocompromised mice by intraspleen injection of immune cells from a naïve primate donor according to the above described method.
- Injection of any immunogenic antigen, infectious agent or parts thereof towards an immune response is wanted.
- Optionally, one or more booster injections of said antigen, infectious agent or parts thereof.
- Isolation of the activated and differentiated primate B cells from the reconstituted immunocompromised mice.

The term "immunogenic" refers to the ability of a substance to cause a humoral and/or cellular response, whether alone or when linked to a carrier, in the presence or absence of an adjuvant.

The present invention also provides a method for producing activated and differentiated primate B cells, starting with reconstituted immunocompromised mice as described above and characterised by the following:

- the primate cells, used in the above described method for reconstitution of immunocompromised mice, originate from a primate donor presenting an immune response towards a well-defined antigen (self or non-self antigen), infectious agent or parts thereof.

- injection of the antigen, the infectious agent or parts thereof into the reconstituted immunocompromised mouse in order to evoke a secondary immune response, and
- the isolation of the activated and differentiated primate B cells from the reconstituted immunocompromised mice.

The reconstituted immunocompromised mice, as described above, offers different possibilities towards the production of different primate, more preferentially human Ig specificity's outside the primate, more preferentially human body. The primate cells and more preferably the human cells producing the Ig specificity's can be isolated from said mice and immortalised either through cellular or molecular biology techniques (Carlsson et al, 1992 and Duchosal et al, 1992), demonstrating the feasibility for the production of primate, more preferably human monoclonal Ig. As can be deduced from the examples, the generation of antigen-specific human Ig in SCID mice, not only depends on the survival of the human cells, but also on the presence of a particular recall antigen at an early but not a late stage of engraftment. Observations in the TM-β1 pretreated NOD-SCID mice indicate that even in the absence of a booster with a recall antigen, high titres of antigen-specific Ig are produced. The production of antigen-specific Ig in the TM-β1 pretreated NOD-SCID mice is, in contrast with the observations in the SCID and TM-β1 pretreated SCID, to a much lesser extent the consequence of the secondary antigen boost.

Therefore, the present invention also relates to a method for producing activated and differentiated primate B cells, as described above, even without the injection of a recall antigen. As a consequence, when injecting immune cells from a donor presenting an immune response towards an unknown antigen, the present invention provides a method, described above, to generate monoclonal antibodies towards said antigen. Said monoclonal antibodies then can be used in the identification of the antigen towards the monoclonal antibodies are evoked.

On the other hand, the present invention also provides a method for analysing whether or not the primate donor presents an immunoresponse towards a well-defined antigen, for example a tumour or autoimmune antigen.

The present invention also concerns a method as described above wherein the isolation of the B lymphocytes is executed between day 3 and day 13 after the cell transfer, preferentially between day 5 and day 12, more preferentially between day 6 and 10. At this stage the B lymphocytes attain a maximum expansion relative to the T cells which later overshadow everything.

As can be deduced from the examples, the present invention also concerns a method as described above wherein the primate donor, more preferably the human donor was immunised with Hepatitis B virus surface antigen (HBsAg).

The HBsAg antibody concentration can be measured by any method known in the prior art, such as by means of the ETI-AB-AUK-3-kit (POO1603, Sorin Biomedica Diagnostics S.p.A., Saluggia, Italy).

The present invention further relates to a method as described above wherein the primate donor was immunised or infected with the HCV virus or parts thereof.

More preferentially, the present invention also relates to a method as described above wherein the human donor is infected with the HCV virus. The HCV antibody concentration can be measured by any method known in the prior art, such as by means of the Innotest HCV Ab IV (Innogenetics NV, Belgium).

The invention also concerns the producing of a primate, more preferentially human monoclonal B cell culture by isolation of B cells from the spleen of a reconstituted immunocompromised mouse as described above and then fusing of these isolated B cells with a (hetero)myeloma.

The success of such a fusion increases considerably when B cells are activated. Until now it has been difficult to activate B cells of primate origin, more preferentially of human origin, in vivo or in vitro before fusion. The method as described above and explained in the examples clearly leads to activation and differentiation of B cells and can thus lead to great success in fusion.

Suitable (hetero)myeloma cell lines are known in the prior art, such as K6H6B5 Cell Line (Caroll et al., 1986) for example.

The present invention also concerns the use of antigen-specific B cells produced in this way to set up gene banks of antigen-specific immunoglobulin mRNAs.

The present invention also concerns primate monoclonal antibodies produced by a primate B cell culture as described above.

The present invention also concerns human monoclonal antibodies produced by a human B cell culture as described above.

The monoclonal antibodies are prepared from the cultures by classic procedures known in the prior art.

The present invention also concerns the use of a primate monoclonal antibody as described above for therapy, diagnosis and/or in vitro imaging.

The present invention also concerns the use of a human monoclonal antibody as described above for therapy, diagnosis and/or in vitro imaging.

More particularly, the present invention also relates to the use of non-human, primate monoclonal antibodies, more preferably chimpanzee monoclonal antibodies as described above for human therapy, diagnosis and/or in vitro imaging.

The present invention also concerns cells or cell products isolated from the reconstituted immunocompromised mice according to this invention.

The present invention also concerns a method of making antibodies wherein these antibodies are isolated from a reconstituted immunocompromised mouse as described above, where this immunocompromised mouse has been immunised with the antigen against which these antibodies are directed.

The present invention also concerns a method of producing monoclonal antibodies where these antibodies are produced by a hybridoma obtained by fusion of B cells isolated from the spleen of a reconstituted immunocompromised mouse, as described above, with a heteromyeloma, and where this immunocompromised mouse has been immunised with the antigen against which these antibodies are directed, and where the hybridoma obtained was selected for the production of antibodies against the antigen used for immunisation.

The present invention also concerns a method, for determining whether a substance possibly evokes an immunogenic response, in which a reconstituted immunocompromised mouse as described above is brought into contact with the substance and the immune response is measured on the basis of the antibodies evoked against the substance.

The present invention also concerns a method for determining whether the primate immune system and more preferentially the human immune system gives a response to a particular infectious agent or parts thereof , characterised by:

- a reconstituted immunocompromised mouse as described above brought into contact with the infectious agent or parts thereof and
- the measuring of the immune response on the basis of the antibodies evoked against this agent, or parts thereof, or the investigation of the pathology of the animal, or the determination based on known markers of the function and activation of primate T, B and NK cells and macrophages.

The present invention also concerns a method for determining whether a substance has an influence on graft versus host disease, where a reconstituted

immunocompromised mouse as described above is brought into contact with the substance and the pathology of the animal is investigated.

The present invention also concerns a method for determining whether a substance influences the primate immune response, where a reconstituted immunocompromised mouse as described above is brought into contact with the substance and the immune response is measured on the basis of the antibodies evoked against a preselected antigen, or the pathology of the animal is investigated, or the function and activation of primate T, B and NK cells and macrophages is determined on the basis of known markers.

The present invention also concerns a method for determining whether a substance influences the primate immune response to a particular infectious agent, where a reconstituted immunocompromised mouse as described above is brought into contact with the substance and the immune response is measured on the basis of the antibodies evoked against the infectious agent, or parts thereof, or the pathology of the animal is investigated, or the function and activation of primate T, B and NK cells and macrophages is determined on the basis of known markers.

The present invention also concerns a method for determining whether a substance influences the primate immune response to a particular primate tumour, where a reconstituted immunocompromised mouse as described above is brought into contact with the substance and the immune response is measured on the basis of the antibodies evoked against the tumour, or the pathology of the animal is investigated, or the function and activation of primate T, B and NK cells and macrophages is determined on the basis of known markers.

The following examples serve to illustrate the invention and may in no way be regarded as limiting.

LEGENDS TO FIGURES

Figure 1: Schematic overview of Example 1.

Figure 2: Overview of the absolute number of B and T cells isolated after 1, 2 and 4 weeks from the peritoneum of TM-β1 pre-treated SCIDs.

Figure 3: Evolution of the number of $CD45^+$ cells in the peritoneum of the reconstituted SCIDs.

Figure 4: Percentage of $CD45^+$ cells of 1, 2 and 4 weeks present in the blood of the TM-β1 pre-treated SCIDs.

Figure 5: Number of $CD45^+$ cells isolated from the spleen of the reconstituted SCIDs, 1, 2 and 4 weeks after cell transfer.

Figure 6: Immunohistological detection of human cells in the spleen (A and B) and liver (C and D) of the HuPBL-SCID mice. The human leukocytes are stained dark via their CD45 marker. No human leukocytes can be detected in the untreated SCIDs (A and C) 4 weeks after reconstitution, whereas large quantities of human cells can be detected in SCIDs which were pre-treated with 1000 μg TM-β1, especially around the blood vessels, but also dispersed in the tissue.

Figure 7: Overview of the IgM and IgG titres in the serum of treated and untreated SCIDs, 1, 2 and 4 weeks after IP reconstruction.

Figure 8: Schematic overview of Example 2.

Figure 9: Ratio of the human B and T cells in the spleen of the HuPBL-SCIDs after 1 and 2 weeks. The exact values can be read off in Table 3.

Figure 10: Overview of the IgG and IgM titres (in ng/ml) found in the serum of the reconstituted SCIDs.

Figure 11: Schematic overview of Example 3.

Figure 12: FL1-FL2 dot plot of the spleen cell population isolated after 24 hours, stained with antibodies directed against the membrane markers CD3, CD14, CD19 (FITC-conjugated), CD16 and CD56 (PE-conjugated). The NK cells ($CD16^-$, $CD56^-$, $CD3^-$, $CD14^-$ and CD19-) were selected in R4 and make up 29.4% of the total leukocyte population.

Figure 13: Progress of intraspleen (IS) reconstitution in TM-β1 treated SCIDs. The number of $CD45^-$ cells isolated from the spleen can be read off from the left Y axis, while the percentages of CD3, CD19 and NK can be read off from the right Y axis.

Figure 14 a: Isotypical control of the HuPBL before sheep erythrocyte rosetting.

Figure 14 b: Staining of the HuPBL before rosetting with antibodies directed against CD19 (FITC-conjugated) and CD3 (PE-conjugated). The $CD19^+B^-$ cells are selected in R2 (11.5%), and the $CD3^+$ T cells are selected in R3 (46.4%).

Figure 15 a: Isotypical control of the HuPBL non-T fraction after sheep erythrocyte rosetting shows that a small amount of aspecific binding occurs.

Figure 15 b: Staining of the HuPBL non-T fraction with antibodies directed against CD3 (PE-conj.) and CD19 (FITC-conj.). From this dot plot we can read off the facts that the fraction of T cells (R4) in this cell mixture is less than 1% and that 18.5% of the non-T fraction is B cells.

Figure 15 c: Staining of the HuPBL non-T fraction with antibodies directed against CD19 (PE-conj.) and CD14 (FITC-conj.). Shows that after rosetting the monocytes (R2) are still present in the cell mixture (46.5%).

Figure 16: Schematic overview of example 4.

Figure 17: Overview of the different types of B cells with the membrane markers that they express.

Figure 18 a: FL3-FSC dot plot of the HuPBL before transplantation. The lymphocyte population was selected in R1 for further analysis.

Figure 18 b: FL1-FL2 dot plot of the lymphocytes selected in Figure 18 a (R1). The HuPBL were stained with antibodies directed against CD19 (PE-conjugated, FL2) and CD20 (FITC-conjugated, FL1). We can see that all $CD19^+$ cells are also $CD20^+$.

Figure 18 c: FL1-FL2 dot plot of the lymphocytes selected in Figure 18 a (R1). The HuPBL were stained with anti-CD19 FITC (FL2) and anti-IgD PE (FL1) antibodies. The naïve B cells are located top right ($CD19^+$, IgD^+) while the memory B cells are located bottom right ($CD19^+$, IgD^-).

Figure 19 a: FL3-FSC dot plot of the cells isolated from the spleen after 3 days. The cells were treated with anti-Mo CD45 cytochrome antibodies so that only human cells were detected in R1.

Figure 19 b: Isotypical control of the human leukocytes selected in Figure 19 a (R1).

Figure 19 c: Staining of the human leukocytes selected in Figure 19 a (R1) with anti-CD19 PE (FL2) and anti-CD20 FITC (FL1) antibodies shows that all $CD19^+B^-$ cells are also $CD20^+$.

Figure 19 d: Staining of the human leukocytes selected in Figure 19 a (R1) with anti-CD19 FITC (FL1) and anti-CD38 PE (FL2) antibodies shows that all the B cells express the activation marker CD38.

Figure 19 e: Staining of the human leukocytes selected in Figure 19 a (R1) with anti-CD19 FITC (FL1) and anti-IgD (FL2) antibodies shows that all the B cells express membrane-anchored Igd.

Figure 19 f: Staining of the human leukocytes selected in Figure 19 a (R1) with anti-CD19 FITC (FL1) and anti-IgD (FL2) antibodies shows that IgG does not occur on the membrane of the B cells.

Figure 20 a: FL3-FSC dot plot of the cells isolated from the spleen after 6 days. The cells were treated with anti-Mo CD45 cytochrome antibodies so that only human cells were detected in R1. We can clearly see that the leukocytes are bigger than normal (higher FSC).

Figure 20 b: FL1-FL2 dot plot of the leukocytes selected in Figure 20 a with anti-CD19 FITC (FL1) antibodies. We can distinguish 2 populations: cells which express the CD19 membrane marker at a high level and cells which express the CD19 only at a low level.

Figure 20 c: Staining of the leukocytes selected in Figure 20 a with anti-CD19 FITC (FL1) and anti-CD38 PE (FL2) antibodies shows that the CD19 low B cells express CD38 at a high level while the CD19 high B cells express CD38 at a low level.

Figure 20 d: FL1-FL2 dot plot of the leukocytes selected in Figure 20 a with anti-CD20 FITC (FL1) antibodies. We can distinguish 2 populations: B cells which are $CD20^+$ and B cells which are $CD20^-$.

Figure 20 e: Staining of the leukocytes selected in Figure 20 a with anti-CD20 FITC (FL1) and anti-CD19 PE (FL2) antibodies shows that the $CD19^{low}$ B cells are $CD20^-$ while the $CD19^{high}$ cells are $CD20^+$.

Figure 21 a: FL1-FL2 dot plot of the leukocytes isolated from the spleen after 2 weeks, stained with anti-CD19 FITC (FL1) antibodies. All the B cells express CD19 at a low level.

Figure 21 b: Staining of the leukocyte population with anti-CD19 FITC (FL2) and anti-IgD PE (FL1) antibodies shows that the majority of the B cells do not have IgD on their membrane.

Figure 21 c: Staining of the leukocytes with anti-CD19 FITC (FL2) and anti-CE38 PE (FL1) antibodies shows that the majority of the B cells express CD38 at a high level.

Figure 22 a: Isotypical control of the leukocytes isolated from the bone marrow after 2 weeks.

Figure 22 b: Staining of the bone marrow cells with anti-CD19 PE and anti-CD20 FITC antibodies shows that the $CD19^{high}$ B cells are $CD20^{+}$ and the $CD19^{low}$ B cells are $CD20^{-}$.

Figure 22 c: Staining of the bone marrow cells with anti-CD38 PE and anti-CD19 FITC antibodies shows that the $CD19^{high}$ B cells express CD38 at a low level and $CD19^{low}$ B cells express CD38 at a high level.

Figure 23:TMß1-treated and irradiated Hu-PBL-SCID mice spontaneously produce high amounts of circulating IgG and IgM. Control SCID and TMß1-pretreated/irradiated SCID or NOD/SCID mice were transplanted in the spleen with $2x10^7$ Hu-PBL, isolated from one donor. At 7 and 14 days post-inoculation, human IgG and IgM concentrations were determined in the plasma. Data represent the mean ± SEM of six mice. * $p<0.05$.

Figure 24: Intraspleen-transplanted human B lymphocytes undergo lymphoblastoid and plasmacytoid differentiation. TMß1-treated and irradiated SCID mice were inoculated in the spleen with $2x10^7$ Hu-PBL. Human hematolymphoid cells were phenotyped by FACS analysis before transplantation in TMß1-treated and irradiated SCID mice .

Dot plot A: FL3-FSC dot plot of the Hu-PBL before transplantation. Before transplantation, analysis was gated on the viable (propidium iodide negative) lymphocyte subpopulation (region outlined in dot plot A).

Dot plot B: Isotypical control of the human lymphocytes selected in the region outlined in dot plot A.

Dot plot C: Staining of the leukocytes selected in dot plot A with anti-CD45 FITC (FL1) and anti-CD71 PE (FL2).

Dot plot D: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD3 PE (FL2).

Dot plot E: Staining of the leukocytes selected in dot plot A with anti-CD20 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot F: Staining of the leukocytes selected in dot plot A with anti-CD38 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot G: Staining of the leukocytes selected in dot plot A with anti-20 FITC (FL1) and anti-CD38 PE (FL2).

Dot plot H: Staining of the leukocytes selected in dot plot A with anti-CD21 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot I: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD23 PE (FL2).

Dot plot J: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD86 PE (FL2).

Dot plot K: Staining of the leukocytes selected in dot plot A with anti-surface IgD FITC (FL1) and anti-CD19 PE (FL2).

Dot plot L: Staining of the leukocytes selected in dot plot A with anti-surface IgM FITC (FL1) and anti-CD19 PE (FL2).

Dot plot M: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-surface IgG PE (FL2).

Dot plot N: Staining of the leukocytes selected in dot plot A with anti-CD40 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot O: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD27 PE (FL2).

Figure 25: Intraspleen-transplanted human B lymphocytes undergo lymphoblastoid and plasmacytoid differentiation. TMß1-treated and irradiated SCID mice were inoculated in the spleen with $2x10^7$ Hu-PBL. Human hematolymphoid cells were phenotyped by FACS analysis before inoculation (Figure 24 A-O). In this figure the human hematolymphoid cells were phenotyped by FACS analysis 7 days after inoculation. After transplantation, analysis was gated on the viable human leukocyte subpopulation (R1 region outlined in dot plot B). Murine lymphoid cells were gated out by staining with Cychrome-conjugated anti-mouse CD45 Ab (dot plot A and B show total spleen leukocytes without and with anti-mouse CD45 Ab respectively).

Dot plot A: FL3-FSC dot plot of the spleen cells 7 days post-transplantation. Analysis was gated on the viable (propidium iodide negative) lymphocyte subpopulation (R1 region outlined).

Dot plot B: FL3-FSC dot plot of the cells isolated from the spleen after 7 days. The cells were treated with anti-Mo CD45 cytochrome antibodies so that only human cells were detected in R1. Analysis was gated on the viable (propidium iodide negative) lymphocyte subpopulation (R1 region outlined).

Dot plot C: Isotypical control of the human lymphocytes selected in the region outlined in dot plot A.

Dot plot D: Staining of the leukocytes selected in dot plot A with anti-CD45 FITC (FL1) and anti-CD71 PE (FL2).

Dot plot E: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD3 PE (FL2).

Dot plot F: Staining of the leukocytes selected in dot plot A with anti-CD20 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot G: Staining of the leukocytes selected in dot plot A with anti-CD38 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot H: Staining of the leukocytes selected in dot plot A with anti-20 FITC (FL1) and anti-CD38 PE (FL2).

Dot plot I: Staining of the leukocytes selected in dot plot A with anti-CD21 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot J: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD23 PE (FL2).

Dot plot K: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD86 PE (FL2).

Dot plot L: Staining of the leukocytes selected in dot plot A with anti-surface IgD FITC (FL1) and anti-CD19 PE (FL2).

Dot plot M: Staining of the leukocytes selected in dot plot A with anti-surface IgM FITC (FL1) and anti-CD19 PE (FL2).

Dot plot N: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-surface IgG PE (FL2).

Dot plot O: Staining of the leukocytes selected in dot plot A with anti-CD40 FITC (FL1) and anti-CD19 PE (FL2).

Dot plot P: Staining of the leukocytes selected in dot plot A with anti-CD19 FITC (FL1) and anti-CD27 PE (FL2).

Figure 26: Phenotypic characterisation of intraspleen-transplanted human $CD4^+$ T-helper lymphocytes. TMß1-treated and irradiated SCID mice were inoculated in the spleen with $2x10^7$ Hu-PBL. Human hematolymphoid cells were phenotyped by FACS analysis before inoculation. Cells were gated as described in Figure 24.

Dot plot A: Isotypical control (IP) of the human leukocytes selected in Figure 24: dot plot A.

Dot plot B: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD3 PE (FL2).

Dot plot C: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD45 RO PE (FL2).

Dot plot D: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD28 PE (FL2).

Dot plot E: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD25 PE (FL2).

Dot plot F: Isotypical ***Dot plot H*** control (IF) of the human leukocytes selected in Figure 24: dot plot A.

Dot plot G: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD38 FITC (FL1) and anti-CD4 PE (FL2).

: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-human HLA DR FITC (FL1) and anti-CD4 PE (FL2).

Figure 27: Phenotypic characterisation of intraspleen-transplanted human $CD4^+$ T-helper lymphocytes. TMß1-treated and irradiated SCID mice were inoculated in the spleen with $2x10^7$ Hu-PBL. Human hematolymphoid cells were phenotyped by FACS analysis before inoculation (figure 26 A-H). In this figure the human hematolymphoid cells were phenotyped by FACS analysis 7 days after inoculation. Cells were gated as described in Figure 25.

Dot plot A: Isotypical control (IP) of the human leukocytes selected in Figure 24: dot plot A.

Dot plot B: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD3 PE (FL2).

Dot plot C: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD45 RO PE (FL2).

Dot plot D: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD28 PE (FL2).

Dot plot E: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD4 FITC (FL1) and anti-CD25 PE (FL2).

Dot plot F: Isotypical control (IF) of the human leukocytes selected in Figure 24: dot plot A.

Dot plot G: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-CD38 FITC (FL1) and anti-CD4 PE (FL2).

Dot plot H: Staining of the leukocytes selected in figure 24 (dot plot A) with anti-human HLA DR FITC (FL1) and anti-CD4 PE (FL2).

EXAMPLES

Example 1: Intraperitoneal reconstitution (IP) of TM-β1 pre-treated SCID mice

1.1 Introduction.

In order to show that TM-β1 pre-treatment of SCID mice leads to better reconstitution, the following experiment was set up (see Figure 1). SCID mice were pre-treated with an intraperitoneal (IP) injection of 0, 100 and 1000 μg TM-β1 antibody (Tanaka et al., 1991), dissolved in 500 μl sterile PBS (9 SCIDs per condition). One day later the treated SCIDs were injected IP with 10^7 Hu-PBL in 1 ml PBS, originating from a healthy donor immunised with Hepatitis B surface Ag (HBsAg). Although the name HuPBL is always used, they are actually Hu-PBMC isolated from a buffy coat or from heparine blood in a manner known in the prior art.

When HuPBL is injected into the spleen the SCID mice are anaesthetised first. After a few minutes the following operation is then commenced. First of all, a cutaneous incision is made in the left side just after the rib cage at the level of the spleen. The peritoneal membrane is then cut open at the same level. The fatty tissue around the spleen is grasped with a pincet and removed from the body. With a 28 Gauge needle the 1 ml syringe is inserted horizontally into the spleen and 50 μl of the cell suspension is injected. The needle is then carefully withdrawn and the peritoneal membrane and the skin are sewn up with a nylon thread (4-0 Dermalon@, 18",45 cm; Davis-Geck, Cyanamid Medical Device Co., Anyang, Korea). Two methods of immunisation were used. In the case of intrasplenic reconstitution the HBsAg (Engerixn4-B, SmithKline Biologicals, Rixensart, Belgium) was injected together with the HuPBL. Another method of immunisation consisted in subcutaneously injecting 2 μg of the HBsAg vaccine (SmithKline Biologicals, Rixensart, Belgium; 1 dose contains 10 μg of antigen in 0.5 ml) at the base of the tail. This was done at the moment when the HuPBL were intraperitoneally injected.

In order to evoke a secondary response the reconstituted mice were subcutaneously immunised with 2 μg HBsAg (Engerix™-B, SmithKline Biologicals, Rixensart, Belgium) daily after HuPBL injection.

After 1, 2 and 4 weeks the Hu-PBL-SCID model was analysed. The percentage and absolute number of human cells ($CD45^+$), B cells ($CD19^+$), T cells ($CD3^+$) and monocytes ($CD14^+$) were determined by FACS analysis in the

peritoneum, spleen and blood. In addition the total quantity of IgG, IgM and the specific antibodies directed against the HBsAg in the serum was determined by ELISA using ETI-AB-AUK-3-kit (POO 3, Sorin, Biomedica Diagnostics S.p.A., Saluggia, Italy).

1.2 Effect of TM-β1 on leukocyte survival in the peritoneum.

Pre-treatment with TM-β1 Ab has a positive effect on the survival of human cells in the peritoneum of the reconstituted mouse (see Table 1). One week after reconstitution a greater quantity of human cells can be detected in the peritoneum of the treated mice than in the peritoneum of untreated SCIDs, 4.3×10^5 and 3×10^6 human leukocytes on average in the SCIDs treated with 100 μg and 1000 μg TM-β1, respectively, and 10^5 leukocytes on average in the untreated SCIDs. After longer incubation periods the effects of the pre-treatment became even clearer. The absolute number of leukocytes present in the peritoneum will thus systematically fall in the untreated mice, 10^4 cells on average after 2 weeks and only 2×10^3 cells after 4 weeks, while in the SCIDs treated with 100 μg TM-β1 the number of leukocytes will reach a maximum of approximately 10^6 human leukocytes after 2 and 4 weeks. The number of leukocytes in the peritoneum of SCIDs treated with 1000 μg TM-β1 decreases, however, 2.6×10^6 and 1.1×10^6 cells after 2 and 4 weeks, respectively. This is probably due to the migration of the leukocytes to the peripheral organs of the reconstituted SCID mice (Figure 3).

Week	TM-β1	Cells isolated in peritoneum						Ig titres in serum	
								IgG	IgM
	μg	%CD45	#CD45	%CD19	#CD19	%CD3	#CD3	(ng/ml)	(ng/ml)
1	0	2.5	10^5	0.43	1.7×10^4	1.27	5×10^4	809	90
	100	11	4.3×10^5	2.17	8.5×10^4	7.06	2.8×10^5	1477	1081
	1000	27	3×10^6	6.5	7.2×10^5	19	2.1×10^6	9247	8748
2	0	0.5	10^4	0.16	3.4×10^3	0.36	7.7×10^3	5918	331
	100	38.3	1.5×10^6	2.9	1.1×10^5	31.2	1.2×10^6	488.224	57.065
	1000	61.6	2.6×10^6	1.5	6.2×10^4	54.2	2.2×10^6	630.677	80.817
4	0	0.1	2×10^3	ND	ND	ND	ND	28.229	1195
	100	25	10^6	0.7	2.8×10^4	23	9.2×10^5	1.333.628	277.476
	1000	45.2	1.1×10^6	2.1	5×10^4	41.4	9.9×10^5	2.220.862	930.874

Table 1: Overview of the average number of human leukocytes isolated from the peritoneum and the average IgG and IgM titres in the serum of TM-β1 pre-treated SCIDs after 1, 2 and 4 weeks.

The percentages of human cells present in the peritoneum of the SCIDs can also be correlated with the pre-treatment thereof. The percentage of $CD45^+$ cells in an untreated mouse will thus be 2.5, 0.5 and 0.1% on average after 1, 2 and 4 weeks, respectively.

In SCIDs pre-treated with 100 μg TM-β1 it is 11, 38.3 and 25% on average after 1, 2 and 4 weeks, respectively, and administration of 1000 μg TM-β1 even gives average percentages of 27, 61.6 and 45.2% $CD45^+$ cells after 1, 2 and 4 weeks, respectively.

Phenotypical analysis of the peritoneal cells shows that, irrespective of the pre-treatment, principally T cells ($CD3^+$) are present after 1 week, and to a lesser extent B cells ($CD19^+$) (see Figure 2). The majority of the T cells isolated are $CD4^+$T helper cells ($CD4^+/CD8^+ = 2.43 \pm 0.70$). The T/B ratio ($3.1 \pm 0.2$) is approximately equivalent to the T/B ratio in the Hu-PBL before transfer to the SCIDs (T/B = 3.62). Human monocytes ($CD14^-$) were not detectable at any time.

After 2 and 4 weeks $CD3^+$ and $CD19^+$ cells are still detectable, but the T/B ratio shifts markedly in favour of the T cells, 10.9 and 35.5 on average after 2 weeks and 32.9 and 19.8 after 4 weeks for 100 μg and 1000 μg TM-β1 administered, respectively.

As the experiment progresses the human B lymphocytes disappear, while there is good survival of the human T cells (see Figure 2).

1.3 Kinetics of the human cell distribution in the Hu-PBL-SCID

The distribution of the human cells in the body of the mice was investigated via FACS analysis of spleen and blood cells. In the control SCIDs, not treated with TM-β1, $CD45^+$ human cells were never detectable in the blood and spleen. In the SCIDs which were injected with a high dose of TM-β1, $CD45^+$ cells were already detectable in the blood and spleen after 1 week. The number of $CD45^+$ cells present in the blood is time- and dose-dependent (see Figure 2) and the presence of human cells in the spleen can be correlated with the number of human cells present in the blood. After 4 weeks even more human $CD45^+$ cells are present in the spleen of SCIDs treated with 1000 μg TM-β1 than were initially injected in the peritoneum (see Figure 5). The human cells which are detected outside the peritoneum are almost all $CD3^+$ T cells.

Immunohistological staining of spleen and liver for $CD45^+$ cells gave equivalent results. In the spleen most of the $CD45^+$ cells are localised around the blood vessels, while a minority are distributed through the tissue (see Figure

6 A and B). In the liver human cells were not detected until after 2 weeks, and then only in SCIDs which were pre-treated with a high dose of TM-β1. Here also most of the human cells are located around the blood vessels (see Figure 6 C and D).

1.4 Effect of TM-β1 on the humoral immune response.

Human IgG and IgM were detectable after 1 week in the serum of all the mice, both treated and untreated. Pre-treatment with 1000 μg of TM-β1 resulted in a higher production of human immunoglobulins. More than 10x higher concentration for IgG and 100x higher for IgM compared with the control SCIDs. The difference between treated and untreated SCIDs became even greater after 2 and 4 weeks. After 4 weeks the total quantity of IgM is 780x higher and the total quantity of IgG 80x higher than in the control SCIDs (see Table 1 and Figure 7). Pre-treatment with TM-β1 thus has a big dose-dependent effect on total Ig production.

A specific secondary immune response was evoked by immunising the SCIDs daily with 2 μg HBsAg after reconstitution. The Hu-PBL were in fact derived from a donor who had already been immunised against the HBsAg (titre = 29,000 IU/L) and the TT(Tetanus Toxin)-Ag (titre = 52,700 IU/L).

In all the mice specific antibodies were found to the HBsAg, but the amount of antibody was clearly dependent on the dose of TM-β1 administered. Compared with untreated mice, SCIDs injected with 1000 μg TM-β1 produce 72x more specific Igs after 2 weeks, and after 4 weeks they produce 36x more HBsAg specific antibodies. Analysis of SCIDs treated with 100 μg TM-β1 gives intermediate values.

Pre-treatment with 1000 μg TM-β1 already yields 1.4x more specific antibodies after 2 weeks than were found in the serum of the donor, and after 4 weeks it even yields 2.6x more (see Table 2).

	TM-β1 (μg)	After 1 week (IU/l)	After 2 weeks (IU/l)	After 4 weeks (IU/l)
Anti-HBsAg titre	0	15	563	2110
	100	<5	2408	17,833
	1000	340	40,333	76,500
Anti-TT titre	0	<50	<50	170
	100	<50	210	100
	1000	<50	190	230

Table 2: Overview of the quantity of human immunoglobulin produced that is specifically directed against the Hepatitis B surface antigen (HBsAg) and the Tetanus Toxin (TT).

1.5 Specificity of the immune response

We can ask ourselves whether the specific humoral immune response obtained in the SCIDs is not in fact due to polyclonal stimulation of the B cells, as it would after all be possible for all the B lymphocytes to be activated by mouse antigens, for example.

In order to investigate this we determined the amount of antibody directed against the tetanus antigen.

The SCIDs were not immunised with the TT (Tetanus Toxin). If the immune response was random, then high titres of TT-specific antibodies should also have been found, but this is not the case (see Table 2).

Only late and low TT-specific antibody production was detectable in both the treated and the untreated SCIDs. Nor is there at any time a visible effect of the amount of TM-β1 administered on the TT-specific Ig titres. We can thus conclude that the humoral immune response was indeed specific.

1.6 Concentration effect of the TM-β1 antibodies administered

It is thus clear that the effect of the TM-β1 antibodies administered is concentration-dependent. Pre-treatment with 1000 μg TM-β1 yields markedly better reconstitution, both on the cellular level (higher number of cells in the peritoneum, blood and spleen) and on the humoral level (higher Ig titres in the plasma). In all subsequent experiments we will therefore eliminate the murine NK cell population by treating each mouse with 1 mg TM-β1 antibodies.

Example 2. Comparison of anti-asialo GM1 and TM-β1 pre-treatment in the intrasplenic (IS) reconstitution of SCID mice

2.1 Introduction

After the highly satisfactory results of TM-β1 pre-treatment in IP reconstitution we are now moving to intrasplenic (IS) reconstitution. If the HuPBL is injected directly into the spleen, the cells are directly located in an organ which supports T cell maturation. Since the injected cells are more closely packed together than with IP reconstitution, the contact between the cells will be improved and a better immune response can probably be evoked. Furthermore, the isolation of cells from the spleen is very practical. There is in fact little cell loss compared with the isolation of cells from the peritoneum. At the same time we will compare the effect of pre-treatment with TM-β1 with pre-treatment with anti-asialo GM1 antibodies (Wako Pure Chemical Industries Ltd, Osaka, Japan). From experiments published in the literature (Murphy et al., 1992; Sandhu et al., 1994) it was apparent that pre-treatment with 20 µl anti-asialo GM1 solution and irradiation with 300 rad 24 hours before transplantation led to optimum reconstitution. We will therefore use this treatment as standard.

For this experiment 18 SCIDs are irradiated with 300 rad before HuPBL transplantation, and of these, 6 SCIDs will be injected IP with 20 µl anti-asialo GM1 solution and 6 SCIDs with 1 mg TM-β1 antibodies. The remaining 6 SCIDs are not given any treatment (see Figure 8).

The HuPBL which are used to reconstitute the SCID mice were isolated by density centrifugation from a buffy coat. Each mouse will be injected intrasplenically with 2×10^7 HuPBL (8.7×10^6 T cells and 1.4×10^6 B cells), suspended in 50 µl sterile PBS. Experiments are undertaken which demonstrate a specific primary response against the Hepatitis B surface antigen. 2 µg HBsAg (SmithKline Biologicals, Rixensart, Belgium) is therefore be injected IS together with the HuPBL.

The success of the reconstitution will be determined after 1 and 2 weeks by FACS analysis on the spleen cell population and by determination of the amount of HuIgG and IgM produced in the blood plasma.

2.2 Analysis of the spleen cell population after 1 week

2.2.1 Absolute and relative number of human leukocytes

FACS analysis of the spleen cell populations 1 week after HuPBL transfer already shows a clear difference between irradiated mice, irradiated mice which were treated with anti-asialo GM1 and irradiated mice which were treated with TM-β1 antibodies (see Table 3). In the SCID spleen of mice which were merely irradiated, only 6.6% of the leukocytes isolated are of human origin ($CD45^+$). In SCIDs which were irradiated and given anti-asialo GM1 treatment, 35.7% on average of the spleen cells present are $CD45^+$, while in TM-β1 pre-treated SCIDs 84.4% of the spleen leukocytes are $CD45^+$.

The absolute number of human leukocytes ($CD45^+$) also differs widely between the various groups of mice. In irradiated mice we recover 7.3×10^4 human leukocytes on average in the spleen, in anti-asialo GM1 treated mice this is already 7.8×10^5 cells, and in the spleen of TM-β1 pre-treated SCIDs 1.9×10^7 human leukocytes are recovered on average.

2.2.2 Phenotyping of the human leukocytes after 1 week

Except in the SCIDs which were only irradiated, the majority of the human leukocytes isolated after 1 week are $CD19^+$ B lymphocytes. Thus, in SCIDs treated with anti-asialo GM1, 60.2% of all the human leukocytes are $CD19^+$ and 27.4% $CD3^+$ T lymphocytes. In TM-β1 treated SCIDs, 79.1% of the human leukocytes are $CD19^-$ and only 14.5% are $CD3^+$.

In all the SCIDs the majority of the T cells are $CD4^+$ T helper cells ($CD4^+/CD8^+$ ratio = 2.9 ± 0.3). The same conclusions can be drawn from the absolute number of human leukocytes found (see Table 3).

Week	Treatment	Cells isolated from the spleen						
		#leukocytes isolated	%CD45	#CD45	%CD19	#CD19	%CD3	#CD3
1	300 rad	1.1×10^6	6.6 (100)	7.3×10^4	2.3 (34.8)	2.6×10^4	4.6 (69.7)	5.1×10^4
	300 rad + anti-asialo	2.1×10^6	35.7 (100)	7.8×10^5	21.5 (60.2)	4.8×10^5	9.8 (27.4)	2.1×10^5
	300 rad + TM-β1	2.2×10^6	84.4 (100)	1.9×10^7	66.8 (79.1)	1.6×10^7	12.2 (14.5)	2.9×10^6
2	300 rad	4.7×10^7	0.5 (100)	1.3×10^5	ND	ND	ND	ND
	300 rad + anti-asialo	2.7×10^7	16.4 (100)	3.8×10^5	6.1 (37.2)	1.3×10^6	10.7 (65.2)	2.5×10^6
	300 rad + TM-β1	5.2×10^6	82.4 (100)	4.2×10^6	5.2 (6.3)	2.8×10^5	77.7 (94.3)	3.9×10^6

Table 3: Overview of the quantity and composition of the leukocytes isolated after 1 and 2 weeks from the spleen of the reconstituted SCIDs. The values in brackets represent the percentages of the subpopulations relative to the total number of human leukocytes (ND = not detectable).

2.3 Analysis of the spleen population after 2 weeks

2.3.1 Absolute and relative number of human leukocytes

Analysis of the spleen population 2 weeks after HuPBL transfer shows that TM-β1 pre-treatment allows better reconstitution than anti-asialo GM1 treatment. Thus, in TM-β1 pre-treated SCIDs 82.4% of the spleen cells present are $CD45^+$ while in anti-asialo GM1 pretreated SCIDs 16.4% of the spleen cells present and in SCIDs which were only irradiated only 0.5% of the spleen cells present are $CD45^+$ (see Table 3).

The effect of irradiation and anti-asialo GM1 treatment has thus clearly ended. In these SCIDs the mouse leukocytes are starting to proliferate again (the absolute number of murine leukocytes in the spleen is increasing) and repopulate the spleen. The TM-β1 antibodies, however, are still active. The

majority of the leukocytes in TM-β1 treated SCIDs are still of human origin (82.4%) and the absolute number of murine leukocytes in the spleen has decreased further (3×10^6 cells after one week and only 1×10^6 cells after 2 weeks).

The difference between the absolute number of human leukocytes isolated from the spleen of TM-β1 and anti-asialo GM1 pre-treated SCIDs is minimal, however, which suggests that the spleen is probably saturated with human leukocytes and these now migrate to the other lymphoid organs of the mouse.

2.3.2 Phenotyping of the human leukocytes after 2 weeks.

Phenotypical analysis of the human leukocytes isolated, 2 weeks after transfer, gives a totally different picture from that after 1 week. The vast majority of the human leukocytes are now $CD3^+$ T cells: 65.2% of all the human leukocytes in anti-asialo GM1 treated SCIDs and as much as 94.3% of all the human leukocytes in TM-β1 pre-treated SCIDs (see Figure 9).

2.4 Humoral immune response.

The conclusions drawn from the FACS analysis of the spleen cells are supported by the human Ig titres found in the SCID plasma. There is in fact also a clear difference in the total quantity of IgG and IgM produced between irradiated, anti-asialo GM1 and TM-βI treated mice, after both 1 and 2 weeks (see Figure 10). Furthermore, after 2 weeks the IgG/IgM ratio in the TM-β1 treated SCIDs (= 9.7) is comparable with the normal IgG/IgM ratio in the serum of a healthy human being.

Example 3. Progress of intraspleen (IS) reconstitution in TM-β1 treated SCIDs

3.1 Introduction

Since in IS reconstitution with TM-β1 pre-treated SCID mice after 1 week 84.4% on average of the leukocytes isolated are $CD45^+$, and 79.1% of them are $CD19^+$, it is useful to investigate the progress of the reconstitution over a very short period, i.e. after 1, 3 and 6 days.

To this end we will reconstitute a total of 12 mice intrasplenically with 2×10^7 cells, isolated from heparin blood. All the SCIDs will be irradiated daily before transplantation with 300 rad and injected intraperitoneally with 1 mg TM-β1 antibodies originating from ascitic fluid. After 1, 3 and 6 days the spleen cells are isolated, in each case from 2 SCIDs, and analysed by means of the FACScan (see Figure 11).

3.2 Analysis of the spleen cell population 1 day after reconstitution

After 1 day an average of 4.4×10^6 leukocytes (human and murine) are isolated from the spleen, 20.2% of them being $CD45^+$.

In order to be able to determine the phenotype of these $CD45^+$ cells, extra FACS stains were applied to the spleen cell suspensions to which anti-mouse-CD45-cytochrome antibodies were added. The murine leukocytes will now fluoresce in FL3, so that we can only analyse the human cells. This reveals to us that 57.7% of the human leukocytes are $CD3^+$ T cells and only 1.4% are $CD19^+$ B cells (see Table 4). A noteworthy feature is the large quantity of human NK cells present, 29.4% of the human leukocytes being NK cells (see Figure 12), whereas the quantity of NK cells in the blood of the donor was only 15.6%. We thus obtain a preferential survival of the human NK cells.

3.3 Analysis of the spleen cell population 3 and 6 days after reconstitution

After 3 days changes in the spleen cell population can already be seen; thus up to 40% of the leukocyte population will be $CD45^+$ and the percentages of both $CD3^+$ and $CD19^+$ cells will increase, 83.3% and 2.7%, respectively. The number of NK cells, however, is progressively decreasing, viz. 13.46% or 6.4×10^6 cells (see Table 4).

It is striking that even though the percentage of $CD45^+$ cells present in the spleen increases, the total number of leukocytes and $CD45^-$ cells decreases. This shows that the mouse leukocytes are progressively eliminated

and a selection of human leukocytes occurs. In actual fact, after 6 days the total quantity of human leukocytes has risen again to 1.24×10^7 cells, making up 82.9% of the total number of leukocytes. The composition of the human leukocytes has also totally changed: the majority of the leukocytes are now $CD19^+$ B cells (80.0%), while the $CD3^+$ T cells are now in the minority (16.34%). Human Natural Killer cells are now also no longer detectable (see Figure 13).

	Cells isolated from the spleen								
day	#leukocytes	%CD45	#CD45	%CD3	#CD3	%CD19	#CD19	%NK	#NK
0			2×10^7	56.5	1.13×10^7	6.5	1.3×10^6	15.6	3.12×10^6
1	4.41×10^6	20.1	8.86×10^5	57.7	5.11×10^5	1.4	1.24×10^4	29.4	2.6×10^5
3	1.25×10^6	39.7	4.96×10^5	83.3	4.13×10^5	2.7	1.34×10^4	13.5	6.7×10^4
6	1.5×10^7	82.9	1.24×10^7	16.3	2.02×10^6	80.2	9.94×10^6	ND	ND

Table 4: Overview of the quantity and composition of the leukocytes isolated after 1, 3 and 6 days from the spleen of the reconstituted SCIDs. The CD3, CD19 and NK percentages are relative to the total number of human leukocytes (ND = not detectable). The values for day 0 represent the composition of the injected cells.

The association between the increase in human leukocytes and the decrease in human NK cells might suggest that the human NK cells are partially responsible for the killing of the transplanted cells. A noteworthy feature is the short division time of the human B cells. Within 3 days the number of B cells increased from 1.34×10^4 cells to 9.94×10^6 cells. This is approximately 9 divisions per 72 hours or approximately 8 hours per division.

Example 4. T cell help is necessary for good reconstitution

An immune response can be evoked in the SCID model in 2 ways:

1. The immune response is specific and the antibody production of the B cells against the antigen requires the help of T cells.
2. The immune response is not specific because of polyclonal activation of the B cells. Additional help from T cells is not necessary here.

In order to investigate whether the immune response really is specific, we will transplant the SCID mice with T cell depleted HuPBL. As a control, SCIDs will also be transplanted with HuPBL from the same donor.

The PBL are depleted of T cells by means of sheep erythrocyte rosetting (a method known in the prior art). Since in this process the HuPBL come into contact with FCS (foetal calf serum), which might serve as an antigen, the PBL which are used to transplant the control SCIDs must undergo the same protocol, but without the addition of sheep blood.

Before rosetting 46.4% of the HuPBL were $CD3^+$ T cells and 11.5% were $CD19^+$ B cells (see Figure 14.a and b). After rosetting we obtain a selective enrichment of B cells and monocytes. The non-T-cell fraction now contains 18.5% B cells, 46.5% monocytes and less than 1% T cells (see Figure 7.a, b and c).

The SCIDs irradiated and pre-treated with anti-asialo GMT are transplanted with 8.25×10^6 non-T HuPBL (18.5% B cells) or with 1.5×10^7 HuPBL (11.5% B cells). Both groups of SCIDs will thus receive the same amounts of B cells (1.65×10^6 cells) (see Figure 16).

From FACS analysis of the spleen leukocytes after 1 week it appears that T lymphocytes are necessary for the survival of the transplanted cells. No surviving human $CD45^+$ cells can in fact be detected in the spleen of SCID mice which have been reconstituted with non-T-PBL. These results are confirmed by the absence of human immunoglobulins in the serum of these SCIDs (see Table 5).

#HuPBL transplanted	analysis of spleen cells # leukocytes	% CD45	# CD45	analysis of serum IgG (ng/ml)	IgM (ng/ml)
1.5×10^7 HuPBL	4.4×10^6	39.9	1.8×10^6	5284	6244
8.25×10^6 non-T-HuPBL	1.7×10^6	0	0	0	0

Table 5: Overview of the leukocytes isolated from the spleen of the SCIDs transplanted with HuPBL and non-T-HuPBL. The corresponding IgG and IgM titres found in the serum of the respective SCIDs are also given.

In the control SCIDs, however, normal quantities of $CD45^+$ cells were recovered (1.65×10^6 $CD45^+$ cells on average). The serum of these SCIDs also contains Ig titres which indicate successful reconstitution, 5284 ng/ml IgG and 6244 ng/ml IgM on average. T cell help is thus necessary for evoking a specific immune response in the SCID model.

Example 5. Subtyping of the B lymphocytes in the HuPBL-SCID model

In the subtyping of the human B lymphocytes isolated from the transplanted SCIDs we will mainly look at the markers CD19, CD20, CD38, CD86 and the membrane-anchored immunoglobulins G, D and M. CD19 and CD20 are both B-cell-specific membrane markers, while CD38 and CD86 are general activation markers.

Before proceeding to the discussion of the phenotype of the B cells isolated, it is useful to give a brief overview of the various types of B cells, with the membrane markers which they express.

In general we can distinguish 5 types of B cells: immature, mature and activated B cells, blast cells and plasma cells. The immature, mature and activated B cells are found in the bloodstream. Blast cells are found in the germinal centres and the lymph nodes. Plasma cells are found in the bone marrow, where they secrete antibodies.

Both the immature and the mature B cells are $CD19^-$, $CD20^-$, $CD38^-$ and $CD86^-$. If the B cells are activated they will weakly express the CD38 markers.

The activated B cells then evolve to blast cells which express the CD38 markers more strongly and become CD86$^+$. The CD20 membrane marker, however, is expressed less. Once the blast cells are differentiated to plasma cells they lose all their membrane markers (CD19, CD20, CD86 and sIg) except the CD38 activation marker, which is highly expressed (see Figure 17).

The SCID mice are reconstituted with 2 types of B cells:

1. Naïve B cells: these are IgD$^+$ and IgM$^-$ (50% of the total B cell population) or are IgD$^+$ and IgM$^+$ (30%).

2. Memory B cells: these are IgD$^-$ and IgM$^+$ (3% of the total B cell population) or IgD$^-$ and IgG$^+$ (5%) or IgD$^-$ and IgA$^+$ (4%).

FACS analysis of the HuPBL shows that all the CD19$^+$ cells are also CD20$^+$ (CD19 and CD20 are both B-cell-specific membrane markers) (see Figure 18.b). The difference between the memory and naïve B cells is clearly visible in Figure 18.c, the HuPBL were stained with antibodies directed against membrane-bound IgD. The memory B cells (IgD$^-$) are situated bottom right, while the naïve B cells (IgD$^+$) are situated top right. Three days after HuPBL transplantation all the CD19$^+$ B cells isolated from the spleen are also CD20$^+$, IgD$^+$ (see Figure 19.c and e) and IgM$^+$ (not shown).

All the B cells now also express the activation marker CD38 (see Figure 19.d) and are IgG$^-$ (see Figure 19.f).

It is striking that after 6 days the lymphocytes are bigger than normal (see Figure 20.a), they have evolved to blast cells. The B cell population can now also be split up into 2 groups, viz. CD19^{high} (high fluorescence) and CD19^{low} (low fluorescence) (see Figure 20.b). Both subpopulations are CD86$^+$ (activation marker) and CD71$^-$ (proliferation marker).

The CD19^{high} B cells are also CD20$^+$ and IgD$^+$ (partial), while they express the CD38 membrane marker at a low level (see Figure 20.c, d and e), they are activated B cells which are not yet differentiated.

The CD19^{low} B cells, however, are CD20$^-$ and IgD$^-$, while they express the CD38 membrane marker at a high level (see Figure 20.c, d and e). They are plasmacytoid B cells.

After 14 days all the B cells are CD19^{low}, IgD$^-$, IgM$^-$, IgG$^-$, CD20$^-$ and CD33^{high}, they have thus all evolved to plasmacytoid cells (see Figure 21.a, b and c).

Analysis of the bone marrow cells 14 days after HuPBL transfer shows that the B cells in the bone marrow have the same phenotype as the B cells which were isolated from the spleen after 1 week (see Figure 22.a, b and c).

Conclusions (example 1 to 5):

When Severe Combined Immunodeficient (SCID) mice are reconstituted intraperitoneally with human peripheral blood leukocytes (HuPBL), the latter will be rejected by the natural immune system (NK cells and macrophages/monocytes) of the SCID mice.

Depletion of the NK population by treatment of the SCID with anti-asialo GM1 antibodies improves the survival of the transplanted cells. A disadvantage is that the anti-asialo GM1 antibodies are only active for 5 to 7 days and, in addition, are very expensive.

The use of TM-β1 antibodies should solve both problems. TM-β1 antibodies are directed against the β subunit of the murine IL-2 receptor and ensure a long-lasting depletion (6 to 7 weeks) of the murine NK population.

In our first experiment we tried to study the effect of TM-β1 pre-treatment on intraperitoneal reconstitution. TM-β1 pre-treatment clearly improved the survival of the injected HuPBL compared with the control SCIDs.

The effect of the pre-treatment was also dose-dependent. The best results were obtained if the SCIDs were pre-treated with 1 mg TM-β1 antibodies. The majority of the human cells isolated from the peritoneum after 1, 2 and 4 weeks are T cells. After a week the human cells spread via the blood to the periphery. After 2 and 4 weeks cells are also detected in the spleen and liver of the reconstituted SCIDs. The survival of the human cells in the HuPBL-SCID can also be clearly correlated with the human Ig titres in the blood of the transplanted mice.

In the IP-HuPBL-SCID model it was also possible to evoke a specific secondary immune response against the Hepatitis B surface Antigen (HBsAg).

After IP reconstitution we tried intrasplenic (IS) reconstitution. If the HuPBL are injected directly into the spleen, the cells are directly located in an organ which supports T cell maturation. Since the injected cells are more closely packed together than with IP reconstitution, the contact between the cells will be improved and a better immune response can probably be evoked.

Furthermore, the isolation of cells from the spleen is very practical. There is in fact little cell loss compared with the isolation of cells from the peritoneum.

If in intrasplenic reconstitution the SCID mice are only irradiated, we obtain poor reconstitution. Few human cells are recovered in the spleen after 1 and 2 weeks. If the SCID mice are additionally treated with anti-asialo GM1 antibodies a clear improvement is seen in terms of survival of the transplanted cells.

If the SCIDs were treated with TM-β1 antibodies, this improvement was even more extreme. The TM-β1 antibodies ensure a higher number of surviving human cells. Since the relative number of human cells is also very high, the number of interfering mouse leukocytes is negligible.

Intrasplenic reconstitution is also better than intraperitoneal reconstitution. Higher numbers of human cells are obtained faster with intrasplenic reconstitution and the humoral immune response is also higher.

It is noteworthy that the composition of the human cells isolated is different. Whereas with IP reconstitution the T cells are the dominant cell population at any time, the T cell/B cell ratio changes as IS reconstitution progresses. Until 3 days after IS transplantation the majority (80%) of the human cells are $CD19^+$ T cells. From that point onwards the B cells start to proliferate with a division time of ±8 hours, so that after 6 days 80% of the cells are human B cells.

After 2 weeks, however, the situation has reversed again. The T cells are now dominant again (95%), while the B cells barely account for 5% of the total human leukocyte population.

A noteworthy feature is the large number of human NK cells present in the spleen one day after transplantation. 29.4% of the human leukocytes are NK cells, while the number of NK cells in the blood of the donor was only 15.6%. We thus obtain a preferential survival of the human NK cells. This is probably because the NK cells are activated by the mouse antigens that are frequently present.

Afterwards the NK population progressively decreases.

The association between the decrease in the NK population and the increase in the human lymphocytes suggests that the human NK cells may be partially responsible for the high cell death of the transplanted cells one day after cell transfer. NK depletion of the HuPBL for transplantation might therefore improve the reconstitution. This still needs to be investigated, however.

T cell help is necessary for good reconstitution. If the HuPBL are depleted of T cells via sheep erythrocyte rosetting and only then are injected

into the spleen of a pre-treated SCID, all the human cells will already be dead after 1 week.

Phenotypical analysis of the HuPBL which were isolated from the spleen of a pre-treated SCID after 1 week shows that some of the B cells injected have differentiated into activated plasmacytoid B cells. These cells are $CD19^{low}$, $CD20^{-}$, $CD38^{high}$ and IgD^{-} and probably produce antibodies. After 2 weeks the majority of the B cells have evolved into these plasmacytoid cells.

It is significant that after 1 week the activated and differentiated B cells can be isolated easily and in high numbers from the spleen of an IS transplanted SCID. This offers good prospects of achieving our ultimate goal, viz. fusing these B cells with a heteromyeloma and thus obtaining monoclonal B cell cultures.

In addition, we can further optimise the HuPBL-SCID model by using NOD/LtSz-SCID mice as recipient. NOD/SC14 mice naturally have a greatly reduced number of NK cells and macrophages. Additional pre-treatment with TM-β1 is expected to lead to even better results in terms of survival of the transplanted cells.

Example 6: Human B cell growth and differentiation in the spleen of immunodeficient mice.

Methods

Mice

C.B.-17 *scid/scid* (SCID) and NOD/LtSz-*scid/scid* (NOD/SCID) mice were bred under sterile conditions and fed at libitum with autoclaved food and water without addition of prophylactic antibiotics. The NOD/SCID strain was free of Emv 30, an endogenous murine ecotropic retrovirus responsible for induction of lethal thymomagenesis (Serreze et al., 1995). Mice were used between 8 and 12 weeks of age. Mice were NK-depleted by a single intraperitoneal injection of 1 mg TM-ß1 in 500 µl phosphate buffered saline (PBS). Sublethal total body irradiation (300 Rad) was done using a linear accelerator (gamma irradiation).

Generation of mononuclear subsets and transplantation

Hu-PBL were isolated from buffy coats or heparinised venous blood by Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway) centrifugation. Depletion of $CD3^+$ T-cells, $CD8^+$ cytotoxic T-cells and $CD16^+/CD56^+$ NK cells was done using respective specific antibody-coated immunomagnetic beads according to the manufacturers instructions (Dynal, Oslo, Norway). For intrasplenic engraftment, animals were anesthesized and a subcostal cutaneous incision was made followed by incisions of the abdominal wall and the peritoneum. The spleen was carefully exposed and injected with 50 µl cellsuspension in PBS. After injection, the spleen was repositioned in the abdominal cavity and the abdominal wall and skin were sutured separately.

Flow cytometry

Analysis of freshly isolated Hu-PBL and single cell suspensions from Hu-PBL-SCID spleen and femoral bone marrow were carried out on a FACscan flow cytometer (Becton Dickinson, San Jose, USA). To overcome the possibility of murine cells staining non-specifically for human markers, they were gated out together with the dead cells (propidium iodide positive) using cychrome-conjugated anti-mouse common leukocyte antigen CD45 (30-F11, Pharmingen, Hamburg, Germany). Human cells were simultaneously stained with antibodies either directly conjugated with fluoresceine isothiocyanate (FITC) or

phycoerythrine (PE) or biotinylated and counterstained with streptavidine-PE (Caltag Laboratories, San Francisco, USA). Isotype controls (X40) and antibodies recognising human CD3 (SK7), CD4 (SK3), CD8 (SK1), CD14 (MoP9), CD19 (4G7), CD20 (L27), CD23 (EBVCS-5), CD25 (2A3), CD27 (L128), CD38 (HB7), CD45 (2D1) and CD45RO (UCHL-1) were from Becton Dickinson. Antibodies specific for human CD16 (3G8), CD38 (HIT2), CD40 (5C3), CD40L (TRAP1), CD56 (B159), CD70 (Ki-24), CD86 (2331), IgD (IA6-2), IgM (G20-127) and IgG (G18-145) were supplied by Pharmingen. Anti-CD21 (BL13) was from Coulter (Miami, USA) and anti-CD71 (T56/14) and anti-HLA-DR (TU36) from Caltag Laboratories.

Cell culture and fusion

A Hu-PBL-SCID spleen cell suspension was prepared. Cells were cultured in 96 well flat-bottomed microculture plates in 200 μl RPMI-1640 medium supplemented with sodium-pyruvate (1 mM), L-glutamine (2 mM), ß mercapto-ethanol ($5x10^{-7}$ M), penicillin (100 U/ml), streptomycin (100 μg/ml), non-essential amino-acids (all from Gibco BRL, Paisley, Scotland) and 10 % Fetal Clone I (Hyclone laboratories, Logan, USA).

For cell fusion, Hu-PBL-SCID spleen cells and K6H6/B5 heteromyeloma cells, washed in calcium-free PBS, were mixed at a 5:1 ratio. Poly-ethylene-glycol 1500 (50 %; Boehringher Mannheim, Germany) was added during 2 minutes and washed away. Fused cells were cultured in 200 μl of the medium mentioned above supplemented with human recombinant insulin (10 μg/ml; Boehringher Mannheim), ouabain (1 μM; Sigma, St.Louis, USA), HAT (Gibco BRL) and 10 % BM Condimed H1 (Boehringher Mannheim).

In vivo immunisation and detection of total and antigen-specific Ig

HBsAg (aluminium hydroxide adsorbed; Engerix-B, Smithkline Biologicals, Rixensart, Belgium) was injected subcutaneously at the hind leg of SCID mice few hours after Hu-PBL transfer. Blood was collected in heparinised tubes by retro-orbital puncture. The in vivo and in vitro production of specific human anti-HBsIg was measured with the ETI-AB-AUK-3 anti-HBs enzyme immunoassay kit (Sorin Biomedica, Saluggia, Italy). Titres are expressed as IU/L (detection limit 5 IU/L).

Two μg of four different HCV antigens (2 core peptides (C1 and C2), 1 NS5A peptide and NS3 protein; all used in the INNO-LIA HCV Ab III (Innogenetics NV, Belgium; described by Maertens et al in Methods in Molecular Medicine, Vol 19: Hepatitis C protocols), was simultaneously IS injected with 2 X 10^7 Hu-PBLs from the HCV infected donor. Blood of the donor was collected in heparinised tubes by retro-orbital puncture. The in vivo and in vitro production of specific human anti-HCV Ig was measured with the Innotest HCV Ab IV (Innogenetics NV., Belgium).

Determination of total human IgG and IgM concentrations in Hu-PBL-SCID plasma was performed by Elisa. Microtiter plates (96-well, Nunc-Immunoplate Maxisorp, Nunc, Roskilde, Denmark) were coated with 100 μl (2 μg/ml PBS) rabbit anti-human IgG (Dako A/S, Glostrup, Denmark) or goat anti-human IgM (Cappel, Organon Teknika Corp., Durham, USA) for 1 hr at 37 °C and subsequently blocked for 2 hrs with 300 μl of 1 % BSA in PBS at 37 °C. In a third step, SCID or NOD-SCID serum or human Ig standards (Behring Diagnostics, Westwood, MA) diluted in PBS containing 0,5 % BSA were added for 1 hr at 37 °C. After 4 washes, bound antibody was detected by incubating the plates with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako) or goat $F(ab')_2$ anti-human IgM (Tago, Biosource, Camarillo, CA) for 1 hr at 37 °C followed by addition of tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature. The enzymatic reaction was stopped with H_2SO_4 and plates were read at 450 nm. The lower detection limits were 10 and 1 ng/ml for IgG and IgM, respectively.

Results and conclusion:

Scid/scid (SCID) mice homozygous for the severe combined immunodeficiency lack functional T and B lymphocytes and are permissive for human immune cell engraftment (Bosma et al., 1983). The common way to construct a human-mouse chimera is by intraperitoneal injection of Hu-PBL (Mosier et al., 1988). Here, we injected $2x10^7$ Hu-PBL directly into the spleen of the animal host (Table 6). Approximately 10^5 human $CD45^-$ cells still resided at the site of injection one week after inoculation. Murine NK cells are known to elaborate an important residual graft resistance in SCID mice (Murphy et al., 1992 and Shpitz et al., 1994). NOD/LtSz-scid/scid (NOD/SCID) mice lack functional T-

and B-cells and have accompanying defects in nonadaptive immunological function, particularly in NK cell activity, inherent to the NOD/Lt strain background (Shultz et al., 1995). As a consequence, NOD/SCID mice supported higher levels of engraftment of intraspleen-transplanted Hu-PBL than did SCID mice (Table 6). Survival of human cells became hundred-fold higher upon pre-treatment of the SCID host with TM-ß1, a rat monoclonal antibody directed against the murine IL-2 receptor ß chain (Tanaka et al., 1993). Blockade of this receptor in vivo selectively eliminates endogenous mouse NK cell activity in various mouse strains and has been shown to severely enhance leukocyte survival also in the intraperitoneal Hu-PBL-SCID model (see example 1). The highest number of human cells was present in the spleen when the recipient SCID mice were not only pretreated with TM-ß1 but also sublethally irradiated before Hu-PBL injection (Table 6). Under these conditions, over 80% of the cells residing in the murine spleen were of human origin. A similar successful Hu-PBL transplantation was achieved in TM-ß1-treated and irradiated NOD/SCID mice.

After intraperitoneal injection of human leukocytes in control or conditioned SCID or NOD/SCID mice, T-cells always constitute the majority of the human cell population present in the peritoneum or the lymphoid organs whilst B-cells only occur in low numbers (Shpitz et al., 1994; Hoffmann Fezer et al., 1992; Hesselton et al., 1995) Here, 7 days after intrasplenic inoculation, B-cells strikingly predominated in the human cell population (Table 6). B-cell predominance was independent of the mouse strain used or the pre-conditioning regimen of the animal host. Highest absolute numbers of human B lymphocytes were present in the spleen of TM-ß1-treated and irradiated SCID or NOD/SCID mice. Human monocytes and NK cells, originally present in the inoculum, were no more detectable and human T-cells constituted only a minor fraction. The immune responsiveness of the engrafted human B-cells was evidenced by early and high spontaneous production of circulating human immunoglobulin (Ig) G and IgM (Figure 23). The Ig repertoire was polyclonal as indicated by normal κ / λ light chain ratios (data not shown). In following experiments, Hu-PBL transfer was done exclusively in TM-ß1-treated and irradiated SCID mice. The engraftment efficiency in these mice was extremely high in that all

Host	Host pretreatment	Total number of spleen leukocytes (murine + human)	%Human CD45$^+$ cells[a]	Absolute number of human leukocytes	% Human CD3$^+$ T cells[b]	% Human CD19$^+$ B cells[b]
SCID	none	$1.4 \pm 0.1 \times 10^7$	0.6 ± 0.1	$8.4 \pm 1.6 \times 10^4$	33.5 ± 1.8	65.2 ± 2.3
NOD/SCID	none	$1.7 \pm 0.1 \times 10^7$	3.6 ± 0.4	$6.6 \pm 0.4 \times 10^5$	32.3 ± 1.8	67.5 ± 2.1
SCID	TMβ1	$3.5 \pm 0.3 \times 10^7$	26.3 ± 1.5	$9.2 \pm 1.1 \times 10^6$	14.3 ± 2.5	80.7 ± 3.2
SCID	TMβ1 + irradiation	$2.5 \pm 0.2 \times 10^7$	84.4 ± 2.6	$2.1 \pm 0.2 \times 10^7$	18.6 ± 2.9	77.3 ± 3.5
NOD/SCID	TMβ1 + irradiation	$2.3 \pm 0.3 \times 10^7$	85.6 ± 4.1	$2.0 \pm 0.3 \times 10^7$	15.6 ± 1.3	79.2 ± 1.9

TABLE 6: Intrasplenic transplantation of Hu-PBL in SCID mice results in predominant B-cell survival.

SCID or NOD/SCID mice were left untreated or were treated with TMβ1 and/or irradiation. The following day, 2×10^7 Hu-PBL, isolated from a single donor, were injected directly into their spleen. Human cell survival at the site of injection and the representation of lymphocyte subsets was assayed by flow cytometry 7 days post-inoculation. Data represent the mean ± SEM of three inoculated mice. Similar results were obtained with Hu-PBL isolated from different blood donors.

[a] percentage of spleen leukocytes (human + murine)

[b] percentage of human leukocytes

Hu-PBL-SCID mice died of xenoreactive graft versus host disease after three to four weeks after Hu-PBL transfer. The extent of human cell engraftment did not vary from donor to donor or from mouse to mouse using the same Hu-PBL donor.

During their presence in the murine spleen, the $CD19^+$ B-lymphocytes became activated and differentiated into lymphoblastoid and plasmacytoid cells. The blastoid phenotype was indicated by the fact that all human $CD45^+$ hematolymphoid cells isolated from the spleen of SCID mice 7 days post-inoculation of Hu-PBL were enlarged (Figure 24, dot plot A vs. Figure 25, dot plot B) and induced to express the transferrin receptor CD71, a marker for proliferation (Judd et al., 1980) (Figure 24, dot plot C vs. Figure 25, dot plot D). Two populations of $CD19^+$ cells could be distinguished: a major cluster characterised by low expression of CD19 ($CD19^{low}$) and a smaller cluster with high CD19 expression levels ($CD19^{high}$) (Figure 25, dot plot E). $CD19^{low}$ cells discontinued the expression of CD20 ($CD20^{neg}$) and became strong CD38 positive ($CD38^{high}$) (Figure 24, dot plot E-G vs. Figure 25, dot plot F-H). CD19 and CD20 are downregulated and CD38 becomes highly expressed upon terminal differentiation of mature B cells into Ig secreting plasma cells (Punnonen et al., 1993; Arpin et al., 1995 and Cerutti et al., 1998). The $CD19^{low}$ $CD20^{neg}$ $CD38^{high}$ cells thus represented B lymphocytes with a plasmacytoid differentiation status. The $CD19^{high}$ cluster still expresses CD20 and only low levels of CD38 (Figure 25, dot plot F-H). This is representative of an activated lymphoblastoid B-cell population. Both lymphoblastoid and plasmacytoid B-cells were further characterised by a disappearance of CD21 and CD23, both known to be gradually lost upon stimulation and terminal differentiation (Boyd et al., 1985), and the appearance of the co-stimulatory molecule CD86 (Hathcock et al., 1996) (Figure 25, dot plot I-K). Part of the lymphoblastoid cells still carried IgG, IgM or IgD on their cell membranes (Figure 25, dot plot L-N). The plasmacytoid cells were further characterised by a disappearance of HLA-DR (data not shown), membrane IgD and CD40 (Figure 25, dot plot O). The absence of CD40 is reported to be specific for terminally differentiated plasma cells (Westendorf et al., 1994). CD5, exposed at high density in some human auto-immune and B cell derived lymphoproliferative

disorders (Warnke et al., 1980), was totally absent on the $CD19^+$ cells present in the murine spleen (data not shown). The different $CD19^+$ phenotypes described above were observable in untreated as well as in TMß1-pretreated and/or irradiated SCID or NOD/SCID mice (data not shown). The questions whether plasmacytoid cells mature out of naive (IgD^+) or memory (IgD^-) B-cells and whether Ig isotype class switch does occur, still remains to be answered.

During the first days after injection of Hu-PBL in the spleen of optimally conditioned SCID mice, there was a substantial loss of T- and B-cells (Table 7). Human NK cells initially survived relatively well and they are believed to negatively modulate human B-cell outgrowth in SCID mice (Carlsson et al., 1992). However, NK depletion of Hu-PBL before intrasplenic injection did not ameliorate but rather slightly reduced the human B-cell engraftment and concomitant human Ig production in our intrasplenic Hu-PBL-SCID model (data not shown). The B-cell differentiation pattern, described above in the case of total Hu-PBL injection, and the Ig κ / λ light chain ratio were not altered when human NK cells were absent. From day 3 on, the CD38, CD71 and CD86 activation markers became apparent on the B lymphocytes (data not shown) going together with a vigorous B-cell growth (Table 7). At day 5, lymphoblastoid and plasmacytoid $CD19^+$ B-cells could be distinguished and were equally represented. As time of transplantation progresses, the plasmacytoid cell population became more and more dominant and represented over 80% of $CD19^+$ B-cells in spleen 14 days after inoculation (data not shown). The predominance of B lymphocytes in the human cell population isolated from the mouse spleen was transient and peaked between day 7 and day 10 post-inoculation. From day 7 on, T-cell growth occurred and the absolute number of harvested human B-cells declined (Table 7). The latter was at least partially due to emigration of B-cells from the spleen as plasmacytoid cells became apparent at extra-splenic sites, especially the bone marrow (data not shown).

The intrasplenic engraftment of B-cells was T-cell dependent. Human $CD19^+$ B-cell survival and human Ig production were hardly detectable in SCID mice reconstituted with T-cell depleted Hu-PBL (data not shown). Herein, $CD4^+$ T-helper (Th) cells rather than $CD8^+$ cytotoxic T-cells did play an important role as

the absence or presence of $CD8^+$ cells in the inoculum did not influence B-cell survival, Ig production or B-cell differentiation (data not shown). During the entire observation period (0-14 days), $CD4^+$ Th-cells represented the major population within the human T-cells (Figure 27). In the inoculum, approximately half of the $CD4^+$ Th-cells demonstrated positivity for the CD45RA isoform that correlates with a naive phenotype (Gray, D., 1993). Until day 3, over 70% of the $CD4^+$ surviving cells retained the unactivated, naive phenotype of donor PBL whilst from day 5 on, the major part (>80%) showed an activated/memory type ($CD45RO^+$) phenotype. Hu-PBL before injection showed minimal staining for activation markers on CD4-positive cells. The activation markers CD25 (IL2 receptor chain), CD38, CD71 and HLA-DR (MHC class II) occurred from day 3 on and coincided with a steady increase in absolute T-cell number.

In the T-cell dependent B-cell activation and Ab response, activated T-helper lymphocytes provide co-stimulatory signals to B lymphocytes during cognate T-B interaction. In vitro experiments indicate that CD40 and CD27 signals involve major steps in this process. First, the ligation of CD40 present on B lymphocytes by the CD40 ligand (CD40L) rapidly and transiently induced on activated Th-cells (Casamayor et al., 1995) provides a first key activation signal required for B-cell activation and proliferation (Clark et al., 1986 and Lane et al., 1995). In the following step, CD70, another type of co-stimulatory molecules present on activated T-cells, seems to actively promote differentiation of activated B-cells into plasma cells through CD27 ligation on the B-cell surface (Tortorella et al., 1995; Jacquot et al., 1997 and Kobata et al., 1995). In addition, cytokines such as IL-2, IL-4, IL-6 and IL-10 are considered to be primarily involved in providing help for B-cells (Banchereau et al., 1994). Both CD40 and CD27 were expressed on the B-cells present in the Hu-PBL-SCID spleen (Figure25). However, the inducible co-stimulatory molecules CD40L or CD70 were never detected within the human cell population throughout the observation period. Probably other co-stimulatory molecules such as the B-cell antigen CD19 (Rickert et al., 1995), the membrane-bound tumour necrosis factor (Aversa et al., 1993) or the signalling lymphocytic activation molecule (SLAM) (Cocks et al., 1995), all known to mediate maturation, proliferation and differentiation of B-cells, may be involved.

Time post inoculation (days)	Total number of spleen leukocytes (murine + human)	% Human CD45⁺ cells[a]	Number of human leukocytes	% Human CD3⁺ T cells[b]	Number of human CD3⁺ T cells	% Human CD19⁺ B cells[b]	Number of human CD19⁺ B cells	% Human CD16⁺CD56⁺ NK cells[b]	% Human CD14⁺ monocytes[b]
0			2.0×10^7	56.5	1.1×10^7	6.5	1.3×10^6	15.6	17.8
1	$5.2 \pm 0.4 \times 10^6$	18.3 ± 1.2	$9.5 \pm 0.4 \times 10^5$	62.5 ± 4.1	$4.6 \pm 0.2 \times 10^5$	2.8 ± 0.1	$2.7 \pm 0.1 \times 10^4$	29.4 ± 3.1	4.3 ± 0.2
3	$1.5 \pm 0.2 \times 10^6$	35.6 ± 2.9	$5.3 \pm 0.2 \times 10^5$	82.6 ± 3.2	$4.2 \pm 0.3 \times 10^5$	4.3 ± 0.2	$2.3 \pm 0.1 \times 10^4$	13.5 ± 1.6	ND
5	$4.8 \pm 0.3 \times 10^6$	52.4 ± 3.3	$2.5 \pm 0.1 \times 10^6$	36.2 ± 2.4	$9.0 \pm 0.5 \times 10^5$	58.3 ± 2.2	$1.5 \pm 0.1 \times 10^6$	ND	ND
7	$1.9 \pm 0.2 \times 10^7$	82.9 ± 3.5	$1.6 \pm 0.1 \times 10^7$	16.2 ± 1.3	$2.6 \pm 0.2 \times 10^6$	82.7 ± 2.1	$1.3 \pm 0.1 \times 10^7$	ND	ND
10	$2.5 \pm 0.2 \times 10^7$	62.4 ± 2.9	$1.5 \pm 0.2 \times 10^7$	21.3 ± 0.6	$3.1 \pm 0.1 \times 10^6$	80.6 ± 1.6	$1.2 \pm 0.1 \times 10^7$	ND	ND
14	$2.8 \pm 0.1 \times 10^7$	51.3 ± 1.8	$1.4 \pm 0.2 \times 10^7$	58.3 ± 2.1	$7.8 \pm 1.4 \times 10^6$	41.0 ± 2.4	$5.7 \pm 0.9 \times 10^6$	ND	ND

TABLE 7: The predominant B-cell survival is transient

TMβ1-pretreated and irradiated SCID mice were transplanted in the spleen with 2×10^7 Hu-PBL. At indicated time points, leukocytes were isolated from the injection site and phenotyped by FACS analysis. First row shows FACS analysis of Hu-PBL before injection. Data represent the mean ± SEM of three reconstituted mice. Similar results were obtained with Hu-PBL isolated from different blood donors.

[a] percentage of total spleen leukocytes (human + murine)

[b] percentage of total human leukocytes

ND: not detectable

Otherwise, presently unknown and yet to be characterised co-stimulatory molecules can make a contribution (Life et al., 1994) and a possible role for cross-reactive membrane molecules present on the murine stromal cells and/or leukocytes remains open.

Human Ag-specific Ig production was studied in optimally conditioned SCID mice engrafted with Hu-PBL isolated from a donor immune to Hepatitis B surface Ag (HBsAg) (6981 IU anti-HBs/L at the moment of donation). In the absence of *in vivo* HBsAg immunisation, only low plasma levels of anti-HBs were detected (Table 8).

Anti-HBs titer (IU/L)	Donor	Hu-PBL-SCID -	Hu-PBL-SCID + HBsAg
Plasma[a]	6981	180 ± 65	2502 ± 407
Culture supernatant[b]	0 (0)	26 ± 12 (43)	320 ± 75 (100)

TABLE 8: In vivo exposition to a recall antigen activates antigen-specific B-cell clones

[a] TM-β1-pretreated and irradiated SCID mice were transplanted in the spleen with 2x107 Hu-PBL isolated from an anti-HBs immune donor. Part of the Hu-PBL-SCID mice were in vivo immunized with 2 mg HBsAg (+HBsAg). Seven days after Hu-PBL transfer, anti-HBs titers were determined in the SCID plasma. Data represent the mean ± SEM of six mice.

[b] Freshly isolated Hu-PBL and human leukocytes isolated from the spleen of SCID mice seven days post Hu-PBL transfer were cultured in vitro without Ag stimulation. Each culture contained 10^5 human $CD19^+$ B-cells. Ten days later, anti-HBs in culture supernatant was estimated. Data represent the mean ± SEM of 96 samples. Data between brackets represent the percentage of cultures with anti-HBs > 10 IU/L.

However, in the plasma of HBsAg-boosted animals, an appropriate secondary immune response was measured within one week after Hu-PBL transfer. Peripheral human B-cells could thus be activated in an Ag-specific manner during SCID engraftment. In analogy, cells isolated from the spleen of the immunised Hu-PBL-SCID mice continued to secrete considerable amounts of anti-HBs Ab *in vitro*. No anti-HBs Abs were produced upon in vitro culture of freshly isolated Hu-PBL neither in absence nor in presence (data not shown) of HBsAg. The latter observation indicates the very low resting level of anti-HBs specific plasma cells in the peripheral blood of a human being.

Spleen leukocytes isolated from *in vivo* immunised Hu-PBL-SCID mice, 7 days post-inoculation, were chemically fused with the heteromyeloma cell-line K6H6/B5 (Carroll et al., 1986). Fused cells were seeded at 10^5 cells in culture medium supplemented with hybridoma growth factors and the selective drugs HAT and ouabain. Ouabain was included for the prevention of anti-HBs production by non-fused human B-cells. After one week, cultures were replenished with fresh medium. Four days later, all cultures contained clusters of viable cells and significant anti-HBs production was measured in 4 % of them.

On the other hand, human Ag-specific Ig production was studied in optimally conditioned NOD-SCID mice engrafted with Hu-PBL isolated from a HCV-seropositive donor (Innotest HCV Ab IV (Innogenetics NV, Belgium) positive: $0.100 < OD < 1.700$). 2 x 10^7 Hu-PBLs isolated from the donor and HCV antigens used in the INNO-LIA HCV Ab III (Innogenetics NV, Belgium) (2 core peptides (c1 and c2), 1 NS5A peptide and NS3 proteins) were simultaneously injected in the spleen of TM-β1 pretreated and irradiated NOD-SCID mice ($N = 6$). After 7 days *in vivo* incubation, the spleen cells were harvested en a spleen cell suspension was prepared. More than 90% of the spleen cells were from human origin and 70% of them were human B cells. At the moment the spleen cells were isolated, the serum of all reconstituted mice revealed reactivity in the INNO-LIA HCV Ab III (Innogenetics NV, Belgium) with the antigens simultaneously injected with the Hu-PBLs. The isolated cells were fused with the heteromyeloma cell-line (K6H6/B5). The fused cells were seeded at 10^5 cells per well in a 96 well flat-bottomed microculture plate (16 plates).

After 1 week, all of the hybridoma mini-cultures contained viable and dividing fusion products. 11 to 13 days after fusion, the supernatant of those cultures was screened on the Innotest HCV Ab IV (Innogenetics NV, Belgium). 24 Innotest HCV Ab IV (Innogenetics NV, Belgium) positive ($0.100 < OD < 1.700$) cultures were subcloned. 18 of them were confirmed on the INNO-LIA HCV Ab III (Innogenetics NV, Belgium).

Monoclonal Abs to recall Ags have been generated from cells isolated from intraperitoneally reconstituted Hu-PBL-SCID through combinatorial gene library generation (Duchosal et al., 1992). This technique remains however highly work intensive in that a high number of clones have to be tested and results in Abs that are cloned as Fab fragments whose biological activity is inferior to that of complete Ig proteins. Other investigators have established cloned cell lines from visible Epstein Barr virus-transformed tumours, growing in the Hu-PBL-SCID, that produce Abs specific for the immunising Ag (Carlsson et al., 1992 and Brams et al., 1998). These tumours however express only monoclonal or oligoclonal B-cell repertoires (Saxon et al., 1991) and the cell lines have rather low Ab production capacity and are generally unstable. The intrasplenic Hu-PBL-SCID model described here may allow us to easily tap the rich memory compartment of human Abs leading to a large repertoire of stable, highly productive hybridomas secreting human mAbs useful in immunohistology and passive immunotherapy.

Example 7: Recall antigen independent immune responses in TMβ1 pretreated NOD-SCID mice.

Twenty-four hours before reconstitution with Hu-PBL, four NOD-SCID mice received a single intraperitoneal injection of 1 mg TMβ1 monoclonal antibody in 500 µl PBS. Peripheral blood mononuclear cells were isolated from heparinized venous blood using Ficoll-Hypaque (density = 1.077 g/ml) (Nycomed Pharma, Oslo, Norway) centrifugation and intra spleen injected (10^7 Hu-PBL per mouse). The Hu-PBLs originate from 2 donors (A and B) who showed humoral immune response to HBsAg, Tetanos Toxoid (TT), Rubella, Measles, Mumps, Varicella zoster (VZV) and Toxoplasmosis. After intra spleen injection of 10^7 Hu-PBL, non of the mice used in the experiment received a recall antigen after engraftment. The *in vivo* production of human antigen-specific Ig in human sera at the moment of cell donation and in chimeric mouse sera at two weeks and four weeks post engraftment, was analysed using the following detection methods:

HBsAg: ETI-AB-AUK-3 anti-HBs enzyme immunoassay kit (Sorin Biomedica, Saluggia, Italy). Titres are expressed as IU/l. Detection limit 5 UI/l.

Tetanos Toxoid (TT): Tetanos Toxoid Sensitive IgG Antibody Kit (Gamma S.A., Angleur, Belgium). Titres are expressed as IU/l. Detection limit 50IU/l.

Rubella: Enzygnost Anti Rubella/IgG (Dade Behring, Belgium). Titres are expressed as IU/ml. Detection limit 4 IU/ml.

Measles: Enzygnost Anti Masern-Virus/IgG (Dade Behring, Belgium). Titres are expressed as IU/ml. Detection limit 150 mIU/ml.

Mumps: Enzygnost Anti Parotitis-Virus/IgG (Dade Behring, Belgium). Titres are expressed as IU/ml. Detection limit 500 IU/ml.

Varicella zoster: Enzygnost Anti VZV/IgG (Dade Behring, Belgium). Titres are expressed as IU/ml. Detection limit 50 mIU/ml.

Toxoplasmosis: Enzygnost Toxoplasmosis/IgG (Dade Behring, Belgium). Titres are expressed as IU/ml. Detection limit 10 IU/ml.

The results, presented in table 9 (donor A) and 10 (donor B), demonstrate that within two weeks after engraftment, a specific Ig immune response is raised

against all the antigens towards the donors showed a humoral immune response. Thus, pretreated NOD-SCID mice tend to produce Ag-specific Ig even without a recall antigen.

Ag-specific Ig in serum of donor A at the moment of donation						
HBsAg IU/L	TT IU/L	Rubella IU/ml	Measles IU/ml	Mumps IU/L	VZV IU/ml	Toxo IU/L
3585	3871	44	340	<230	130	11

Mouse	Human Ag-specific Ig in mouse serum: Week 2								
	IgG ng/ml	IgM ng/ml	HBsAg IU/L	TT IU/L	Rubella IU/ml	Measles IU/ml	Mumps IU/L	VZV IU/ml	Toxo IU/L
NOD TMβ1 1	1484653	205942	2561	433	107	170	<230	51	
NOD TMβ1 2	1586925	167610	2753	209	15	180	<230	110	
NOD TMβ1 3	1698375	214555	3105	400	<15	390	<230	55	
NOD TMβ1 4	1344268	216546	3251	352	15	330	<230	53	
	Human Ag-specific Ig in mouse serum: Week 4								
NOD TMβ1 1	3161555	2608752	26345	731	<15	320	<230	200	<6
NOD TMβ1 2	1354891	801712	1157	311	<15	220	<230	<50	<6
NOD TMβ1 3	died								
NOD TMβ1 4	died								

Table 9: Detection of *in vivo* production of human antigen-specific Ig in serum of human donor A at the moment of cell donation and in chimeric mouse (NOD-SCID TMβ1) sera two and four weeks after engraftment of Hu-PBLs from donor A.

Ag-specific Ig in serum of donor B at the moment of donation						
HBsAg IU/L	TT IU/L	Rubella IU/ml	Measles IU/ml	Mumps IU/L	VZV IU/ml	Toxo IU/L
3603	2422	65	5600	480	970	340

Mouse	Human Ag-specific Ig in mouse serum: Week 2								
	IgG Ng/ml	IgM ng/ml	HBsAg IU/L	TT IU/L	Rubella IU/ml	Measles IU/ml	Mumps IU/L	VZV IU/ml	Toxo IU/L
NOD TMβ1 1	1551194	250826	2320	384	15	1200	<230	62	<6
NOD TMβ1 2	2390603	678104	3368	571	17	3200	<230	360	26
NOD TMβ1 3	died								
NOD TMβ1 4	died								
	Human Ag-specific Ig in mouse serum: Week 4								
NOD TMβ1 1	688192	159521	6921	236	<15	2400	<230	100	6
NOD TMβ1 2	366528	56687	2625	108	<15	740	<230	<50	<6
NOD TMβ1 3	died								
NOD TMβ1 4	died								

Table 10: Detection of *in vivo* production of human antigen-specific Ig in serum of human donor B at the moment of cell donation and in chimeric mouse (NOD-SCID TMβ1) sera two and four weeks after engraftment of Hu-PBLs from donor B.

Example 8: Generation of monoclonal antibodies directed against an antigen for which no pre-existing immunity is detected in the donor.

Chimpanzees are immunised with the envelope protein 1 (E1) of hepatitis C virus (HCV) (WO96/04385). Each injection consists of 50 μg E1 adjuvanted with Ribi according to the manufacturers protocol. Two weeks after three or six immunisations, with a three week interval between immunisations, PBMC are isolated of which $1x10^7$ cells are injected in the spleen of TM-β1 pre-treated and irradiated NOD-SCID mice, as described in example 6.

After 7 days, the spleen is removed, the remaining cells are isolated and fused with the heteromyeloma K6H6/B5. Fused cells are cultured as described in example 6. After 10 to 14 days, the fusion is screened for the presence of E1-antibody secreting wells by indirect ELISA: specific antibodies binding to solid phase E1 are detected using anti-human IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for colour development. Positive wells are further expanded and subcloned as described in example 6.

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CLAIMS

1. Method for reconstituting immunocompromised mice with primate cells characterised by the intra-spleen injection of primate cells in said mice.

2. Method according to claim 1, wherein the immunocompromised mice are pre-treated by administration of an antibody directed to the mice natural killer cells and/or gamma irradiation.

3. Method according to claim 1 or 2, wherein the said mice are depleted of functional T and B lymphocytes.

4. Method according to claim 1 or 3, wherein the said mice are *scid/scid* (SCID) mice or NOD/LtSz-*scid/scid* (NOD-SCID) mice.

5. Method according to claim 1 to 4, wherein said primate cells originate from a primate donor who has been immunised with a well-defined antigen or infectious agent.

6. Method according to claim 1 to 5, wherein the primate donor is a human.

7. Method according to claim 1 to 6, wherein said antibody is directed to the beta chain of the mouse interleukin 2 (IL-2) receptor.

8. Method according to claim 1 to 7, wherein said antibody directed to the beta chain of the mouse interleukin 2 (IL-2) receptor is a monoclonal antibody.

9. Method according to claim 1 to 8, wherein said monoclonal antibody is the TM-β1 monoclonal antibody.

10. Method according to claim 1 to 9, wherein the primate cells are human peripheral blood leukocytes (HuPBL's).

11. Method according to claims 1 to 10, wherein the number of HuPBL's used for reconstitution lies preferentially between $1x10^7$ and $5 x10^7$ and is more preferentially between $1 x10^7$ and $3 x10^7$, and preferably $2 x10^7$.

12. An immunocompromised mouse reconstituted with primate cells by the method of Claims 1 to 11.

13. An immunocompromised mouse reconstituted with HuPBLs which is characterised by the fact that 6 to 7 days after the reconstitution of the mouse with human cells up to 70 to 80% of the human cells found in the spleen are CD19+ B cells.

14. A reconstituted immunocompromised mouse according to Claim 12 or 13 for use as an animal model system.

15. Use of a reconstituted immunocompromised mouse according to claim 12 or 13 for the study of the immunobiology of primate hemopoietic and lymphomonocytic tissues.

16. Use of a reconstituted immunocompromised mouse according to claim 12 or 13 for the study of human or animal viruses and more preferentially B lymphotropic human or animal viruses.

17. Primate monoclonal B cell culture produced by isolating the B cells of a reconstituted immunocompromised mouse according to Claim 12 or 13 and then immortalising these B cells by fusion with a heteromyeloma.

18. Primate monoclonal antibodies produced by a primate monoclonal B cell culture according to claim 17.

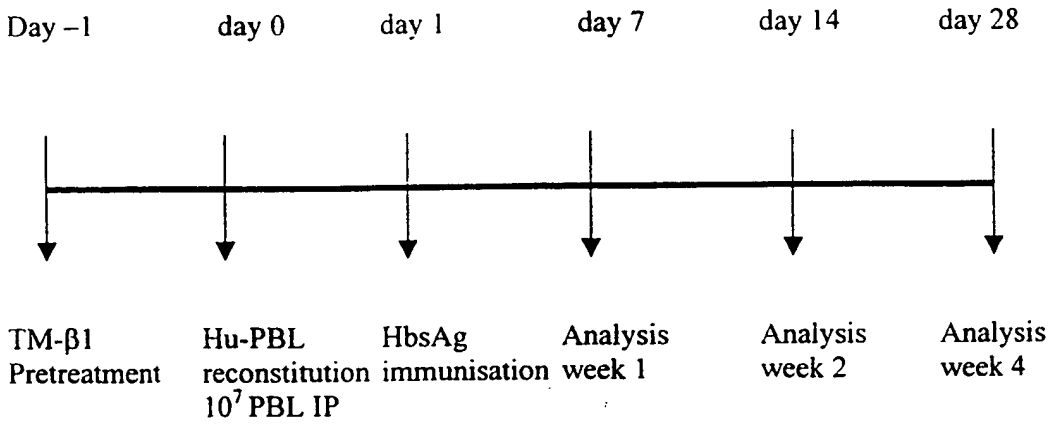
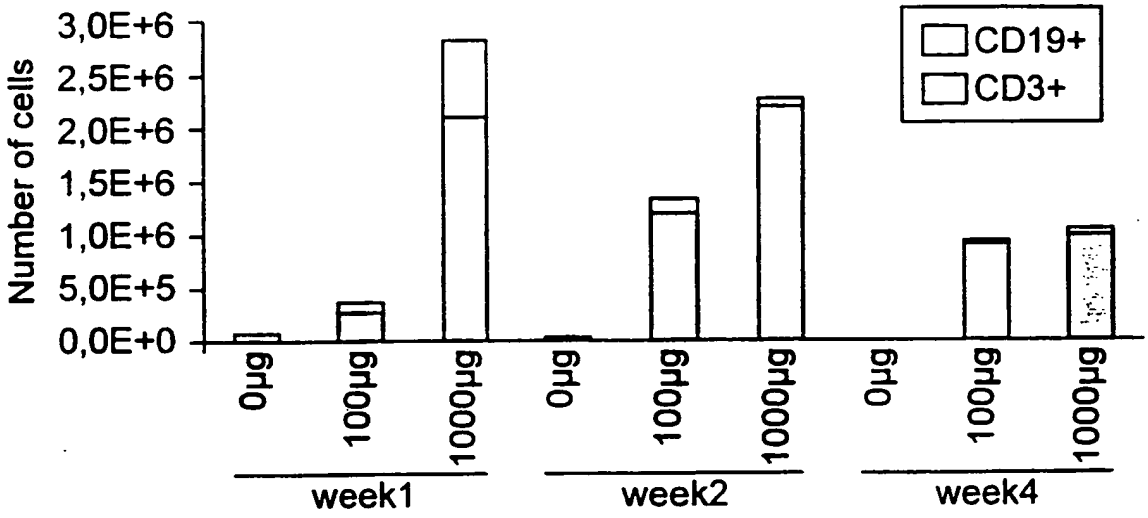


Fig. 1



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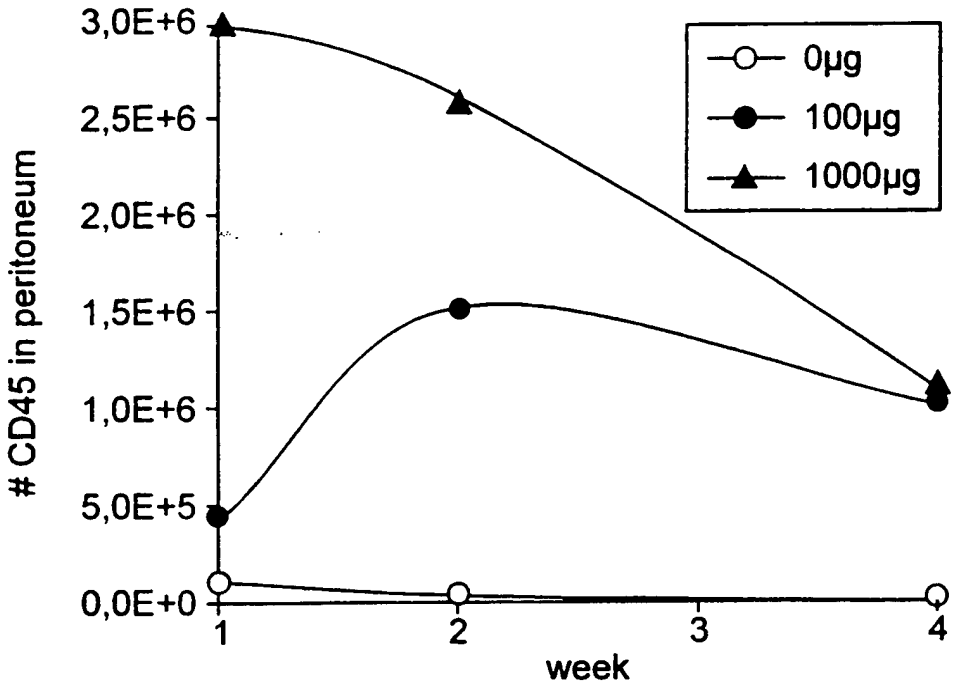

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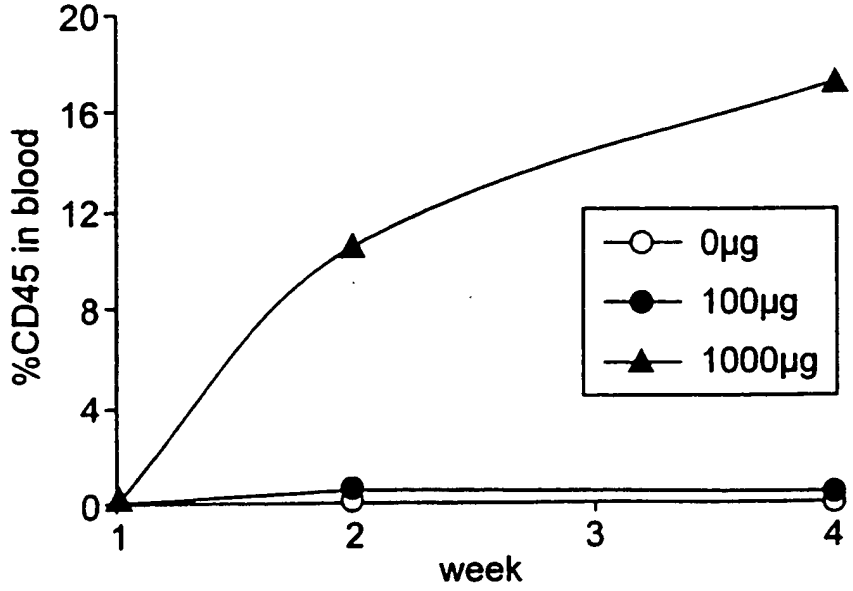

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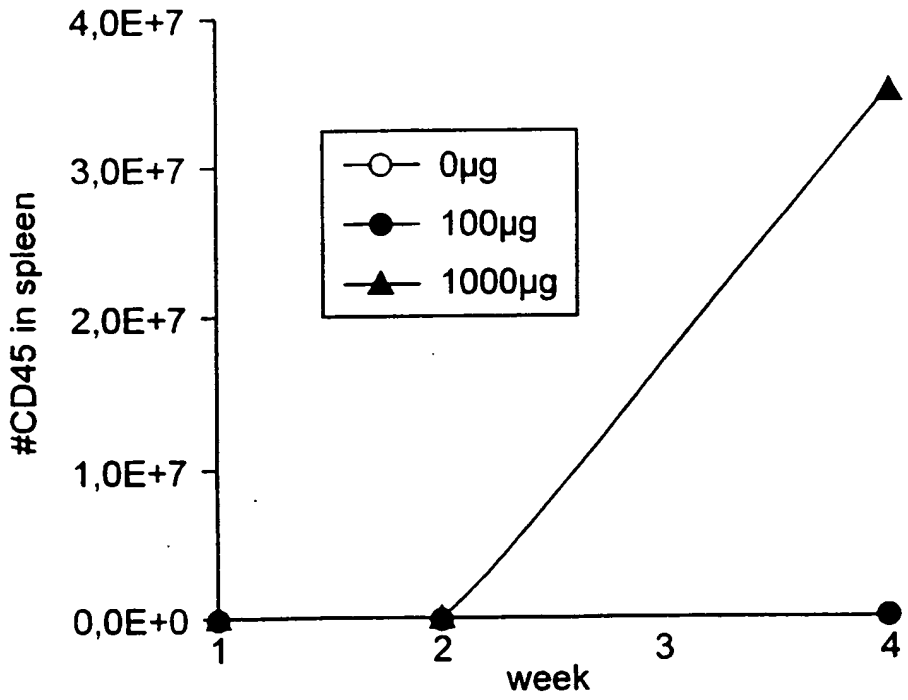

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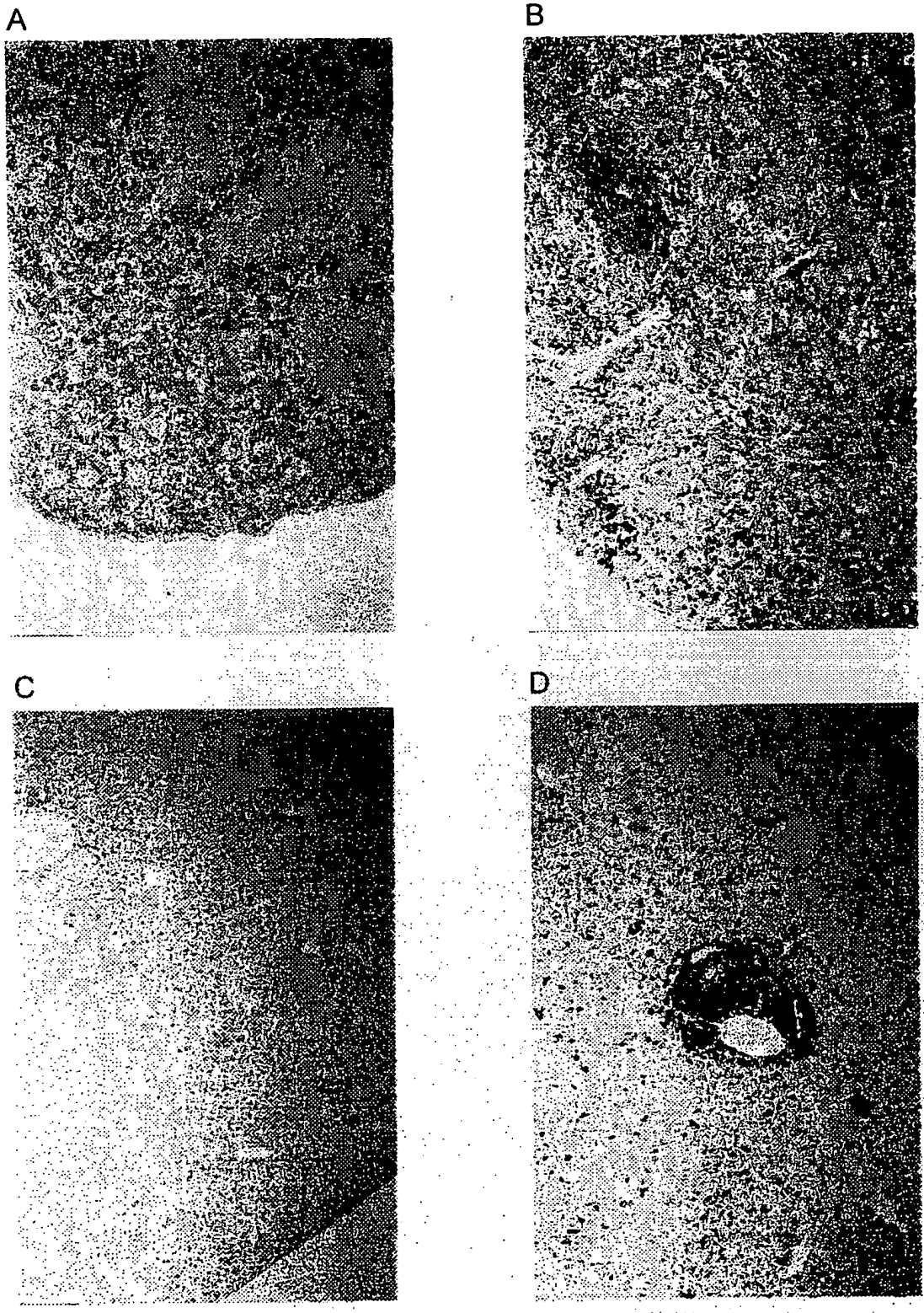

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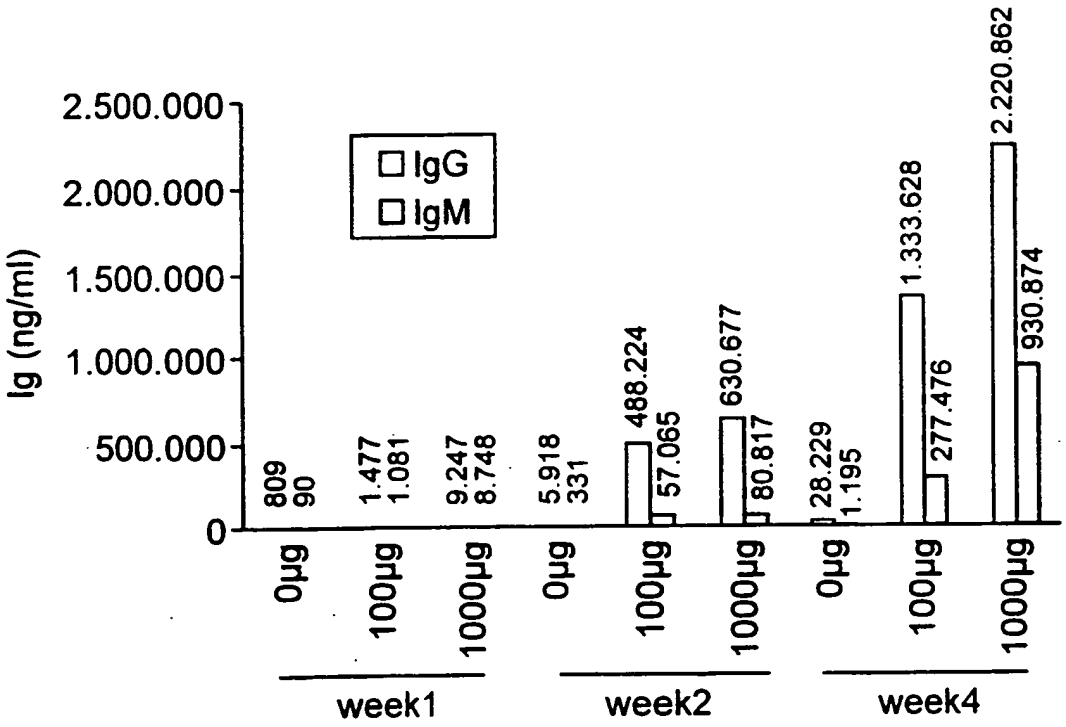

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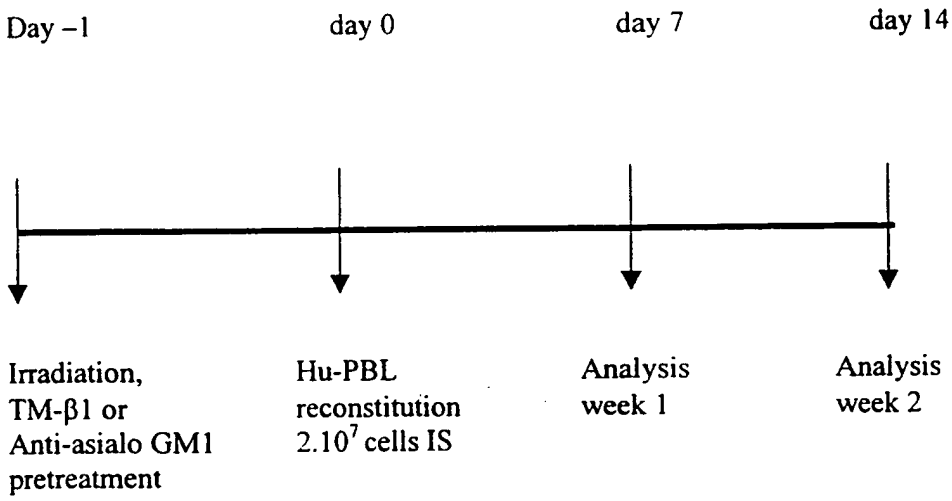

Fig. 8

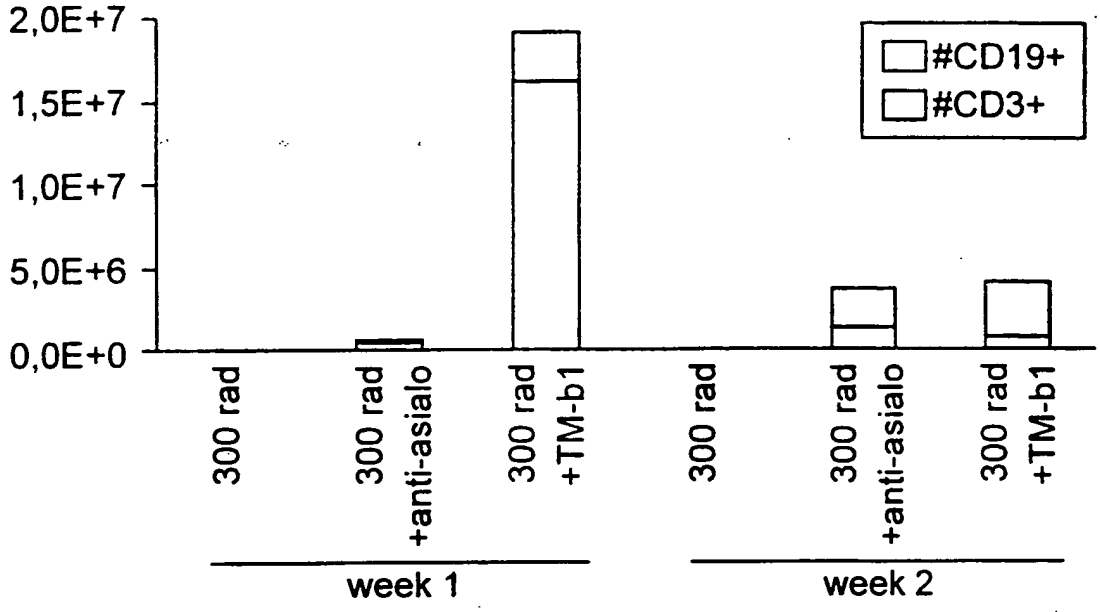

Fig. 9

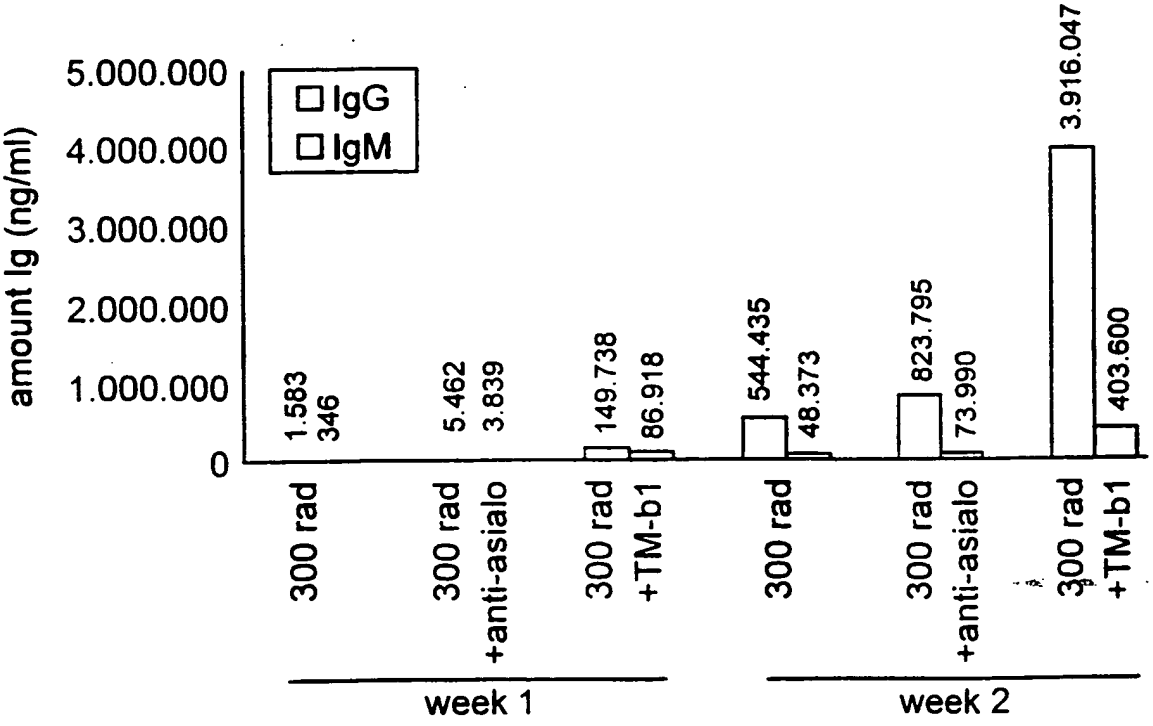

Fig. 10

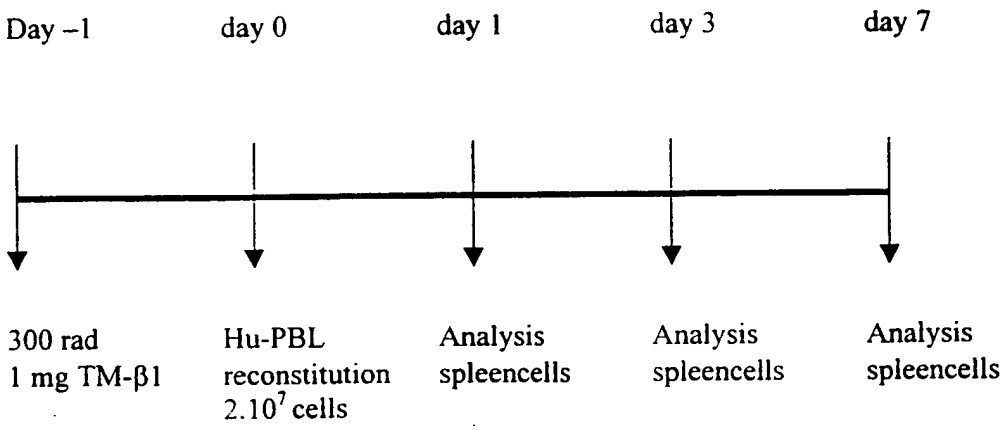

Fig. 11

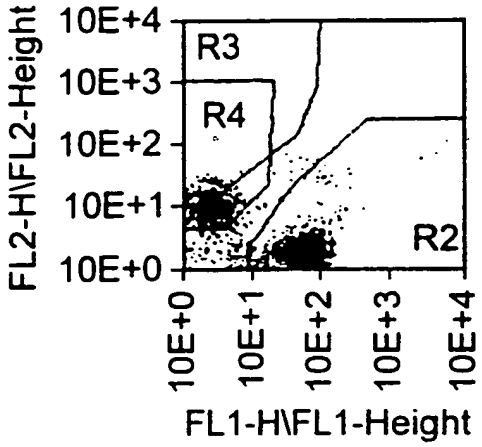

Fig. 12

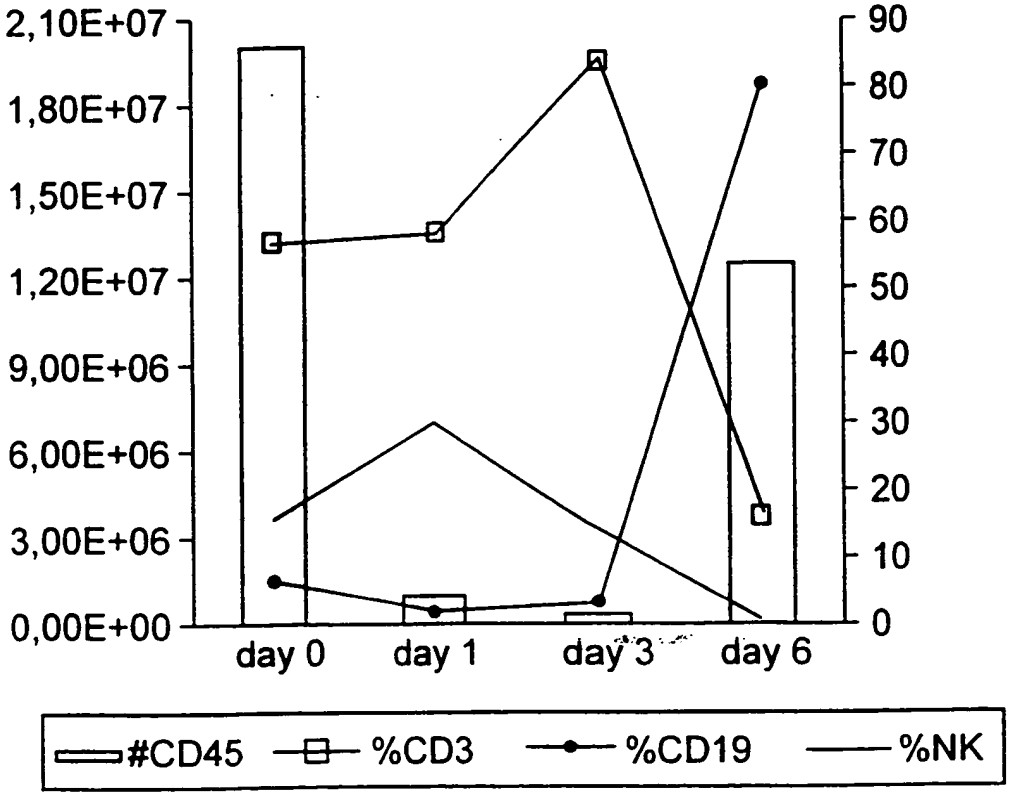

Fig. 13

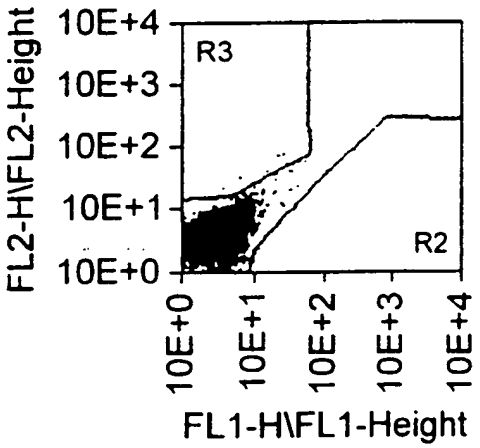

Fig. 14a

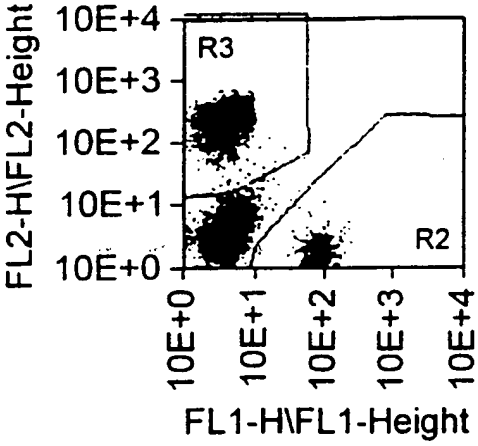

Fig. 14b

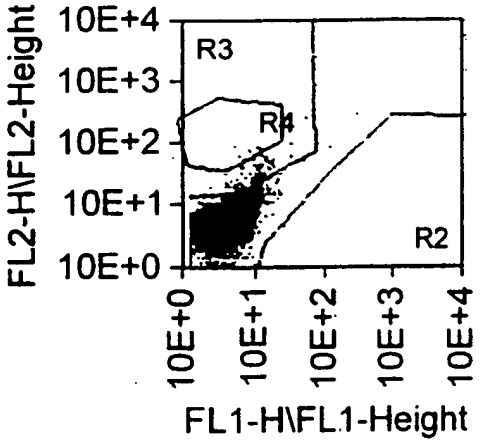

Fig. 15a

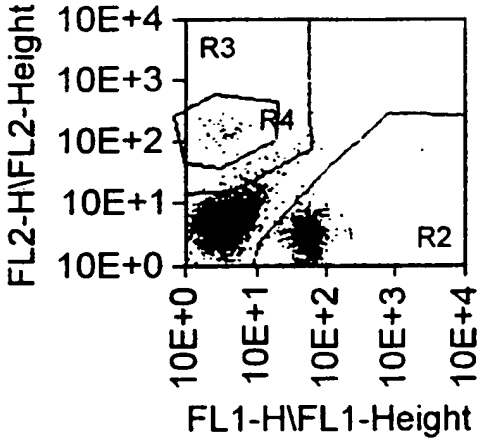

Fig. 15b

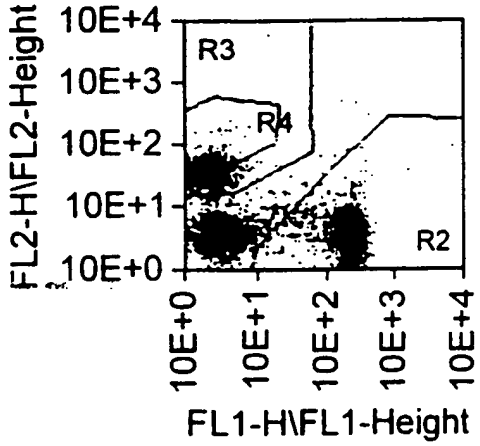

Fig. 15c

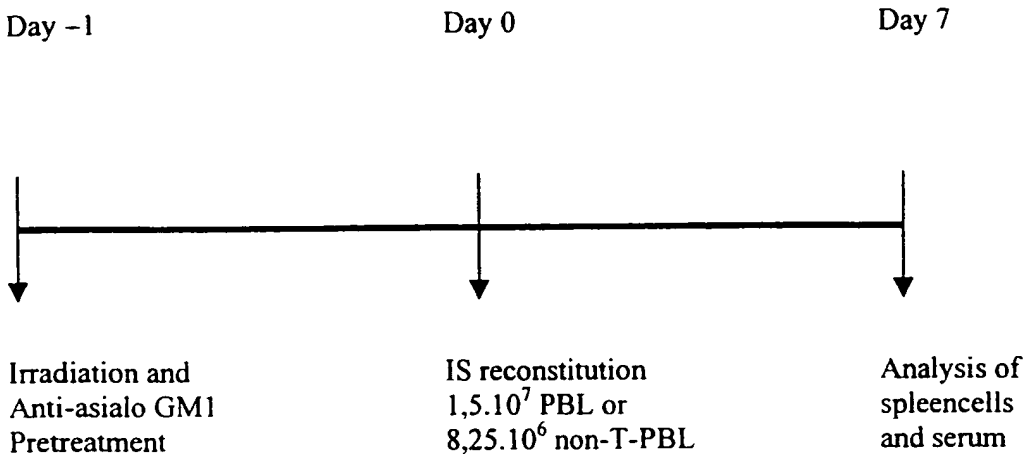

Fig. 16

	Peripheral blood			Germinal centres And Lymphnodes	bonemarrow
Membrane markers	Immature B-cells	Mature B-cellen	Activated B-cellen	Blast cells	Plasma cells
sIg	+	+	+	+/-	-
CD19	+	+	+	+	-
CD20	+	+	+	+/-	-
CD38	-	-	weak	Weak/+	+
CD86	-	-	-	+	-

Fig. 17

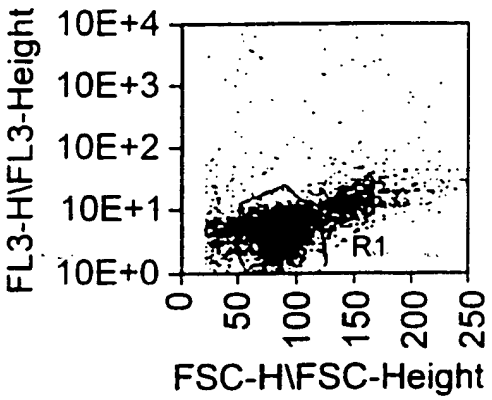

Fig. 18a

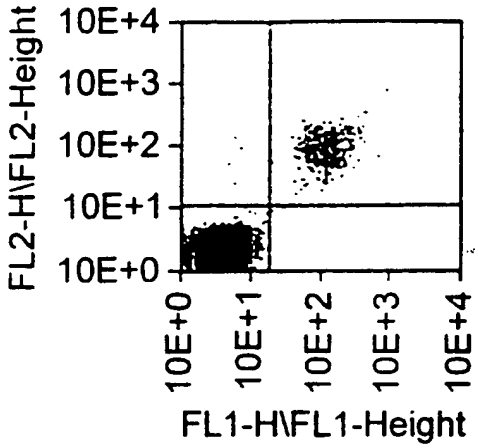

Fig. 18b

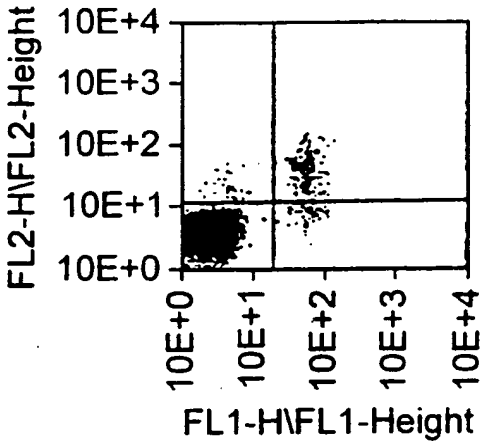

Fig. 18c

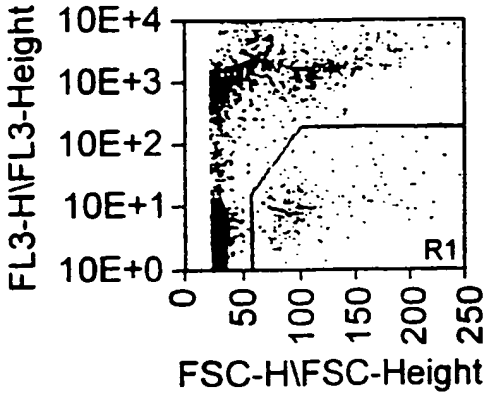

Fig. 19a

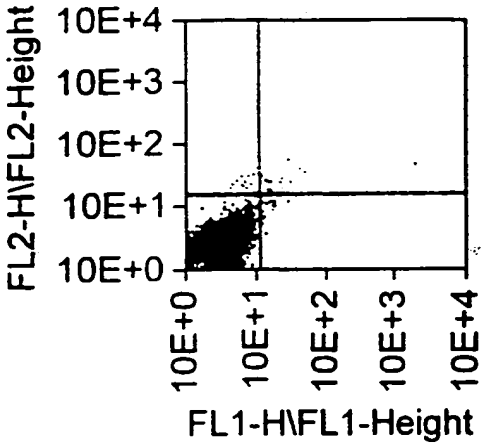

Fig. 19b

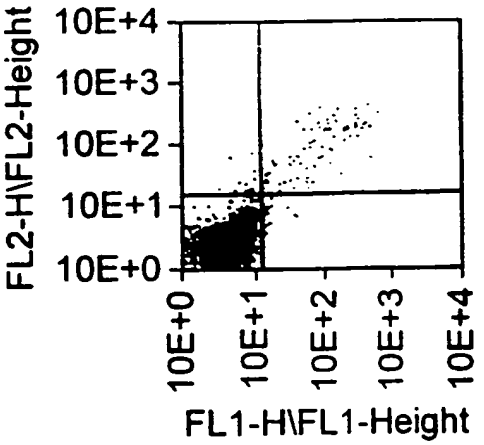

Fig. 19c

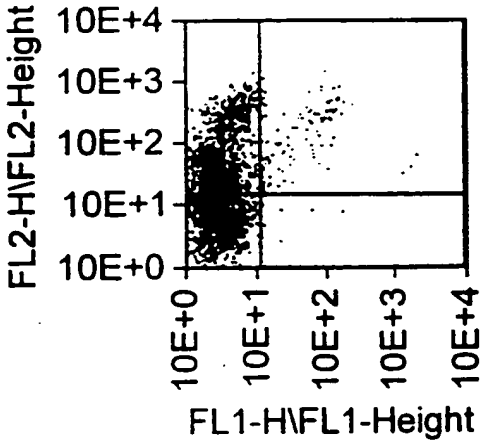

Fig. 19d

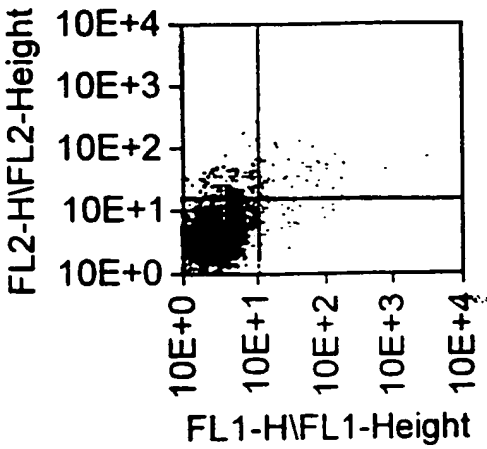

Fig. 19e

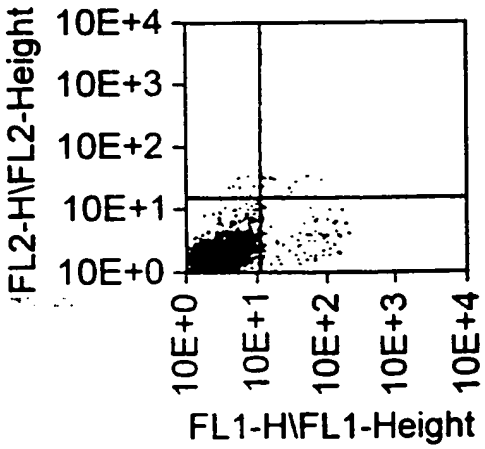

Fig. 19f

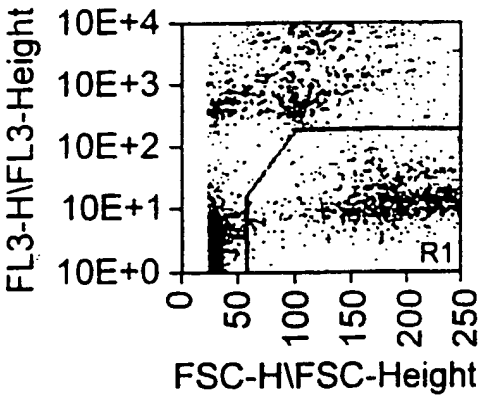

Fig. 20a

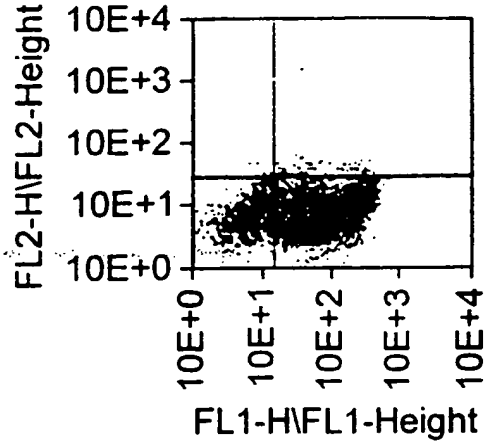

Fig. 20b

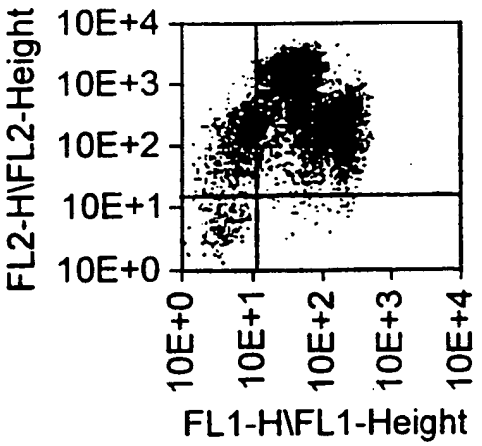

Fig. 20c

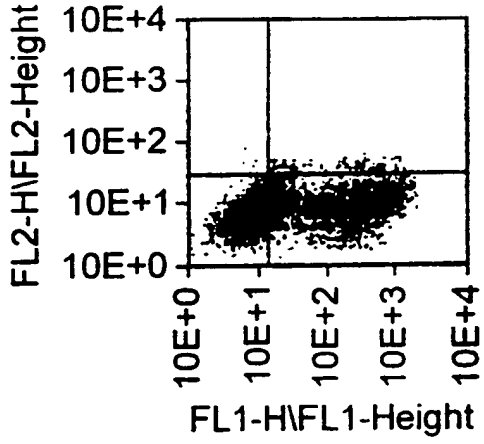

Fig. 20d

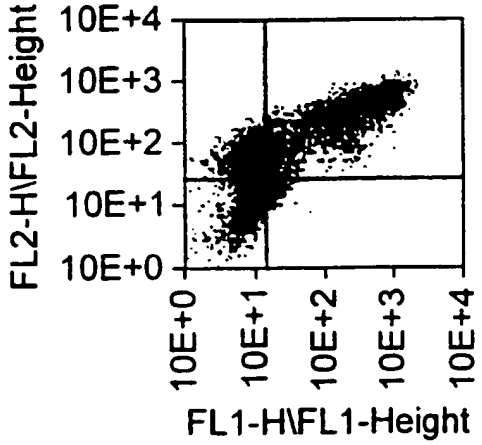

Fig. 20e

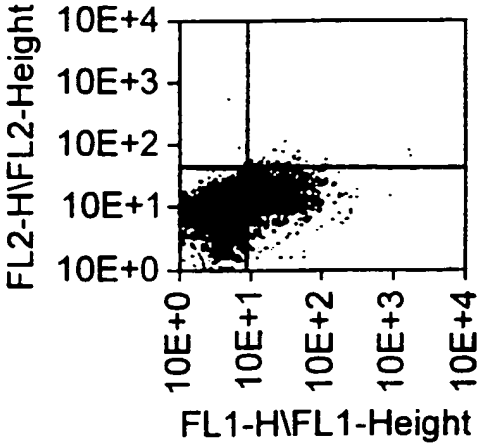

Fig. 21a

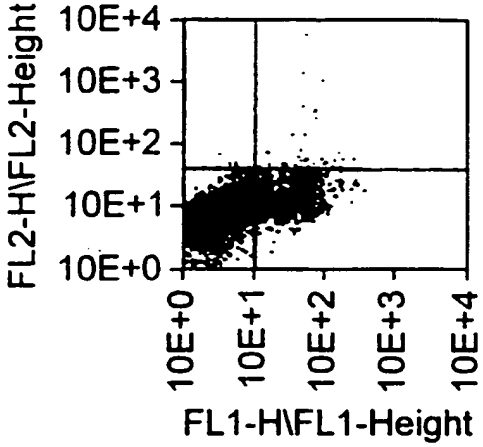

Fig. 21b

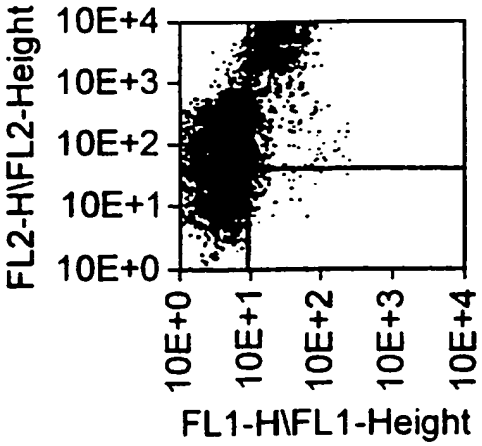

Fig. 21c

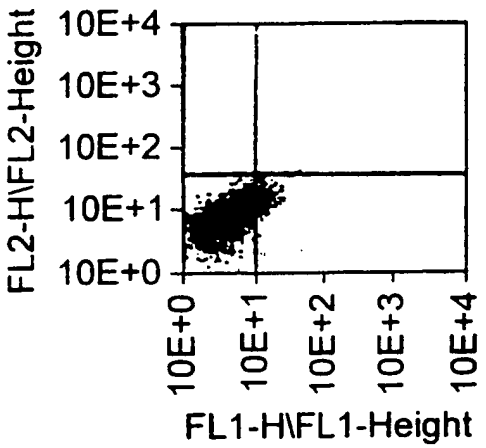

Fig. 22a

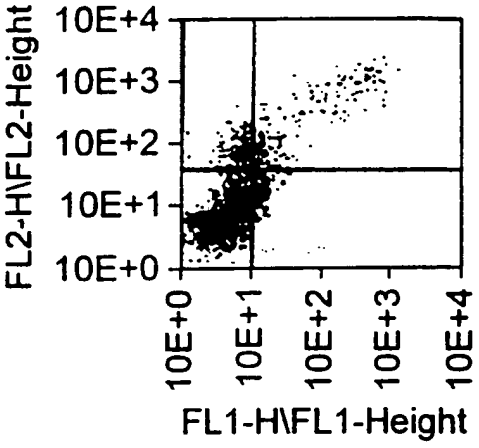

Fig. 22b

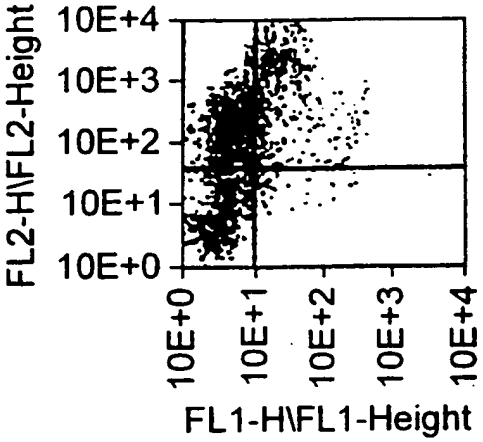

Fig. 22c

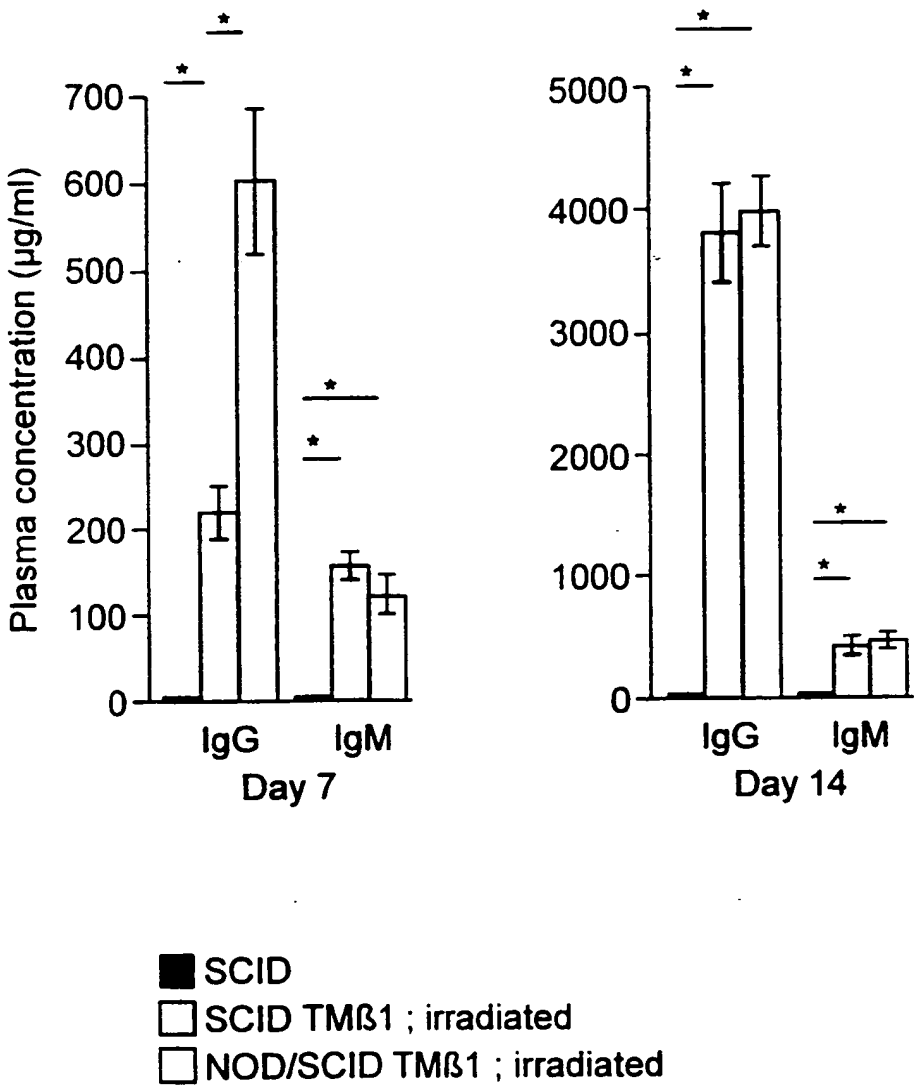

Fig. 23

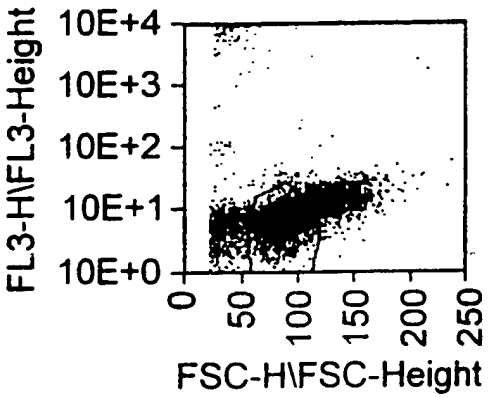

Fig. 24a

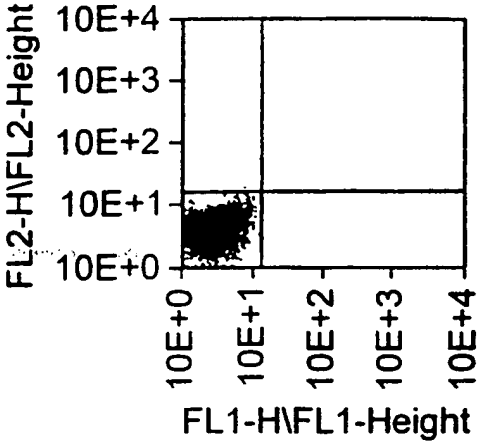

Fig. 24b

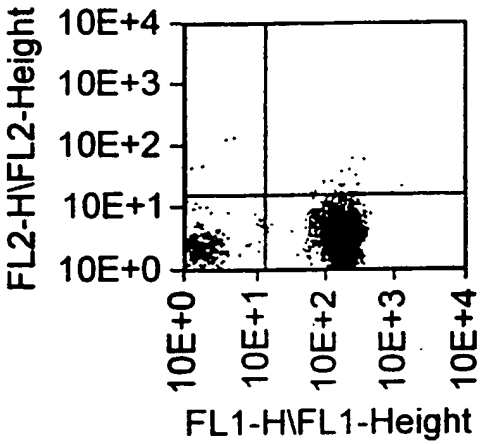

Fig. 124c

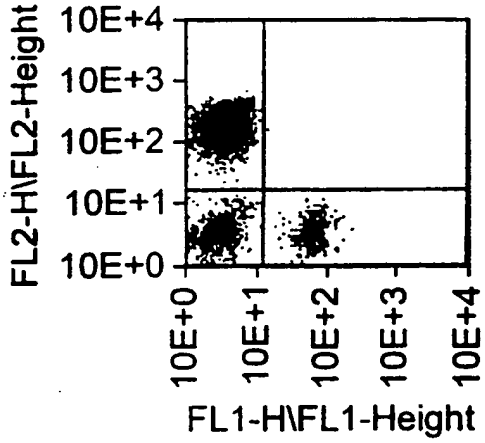

Fig. 24d

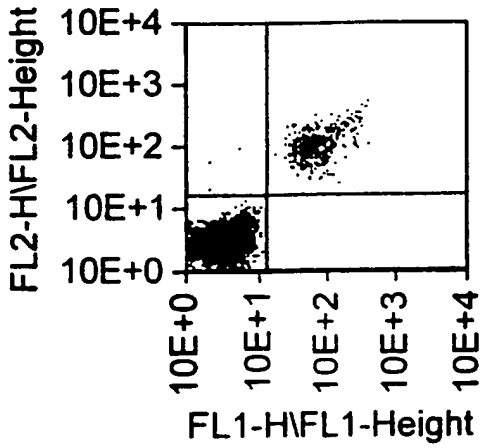

Fig. 24e

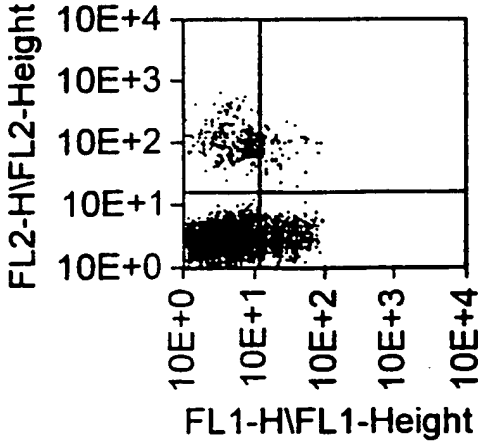

Fig. 24f

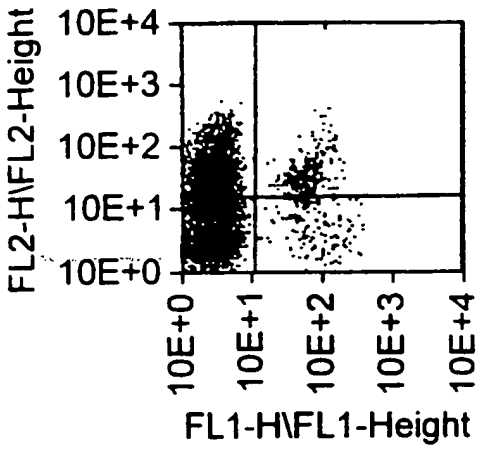

Fig. 24g

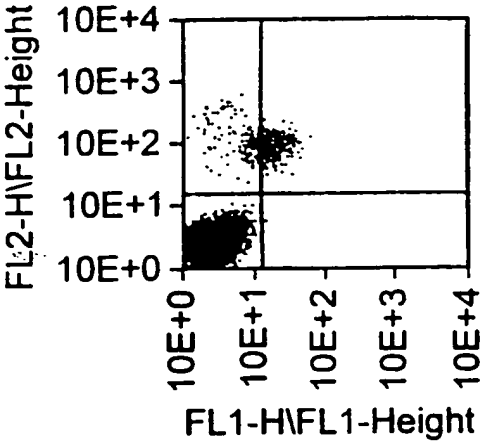

Fig. 24h

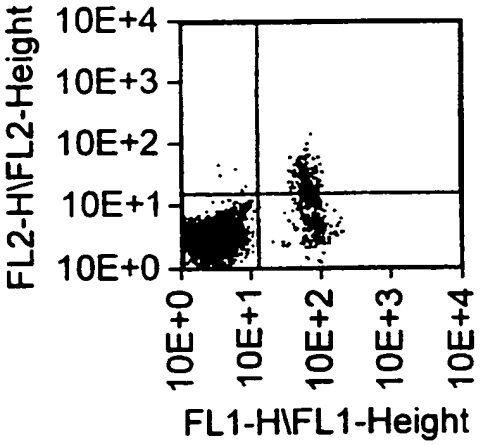

Fig. 24i

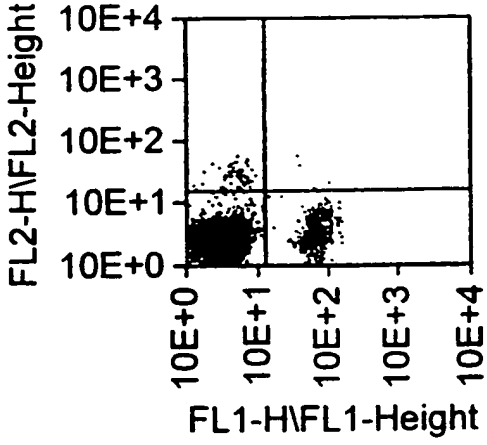

Fig. 24j

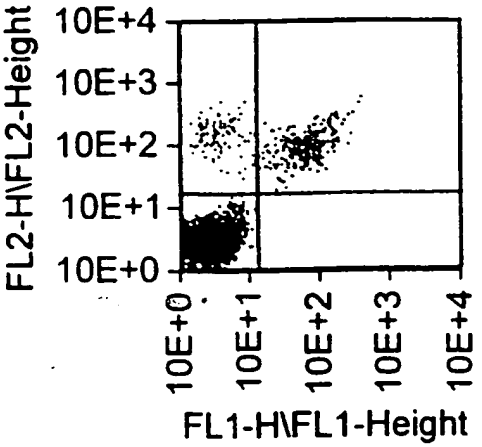

Fig. 24k

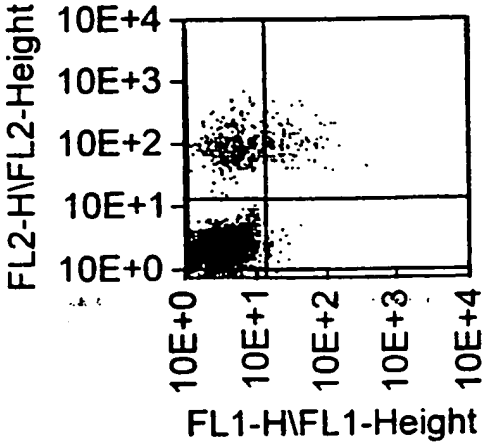

Fig. 24l

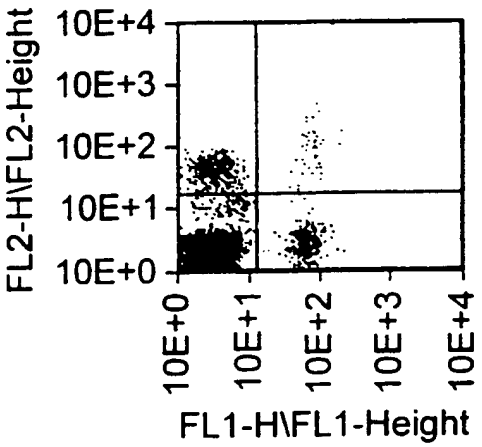

Fig. 24m

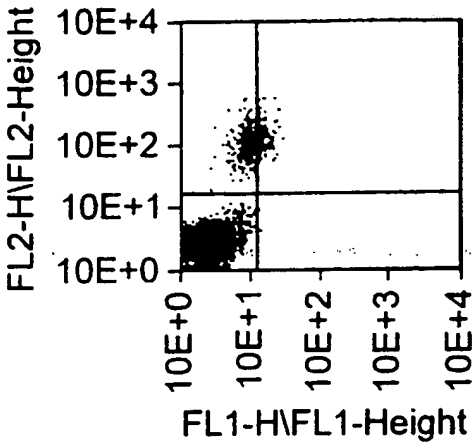

Fig. 24n

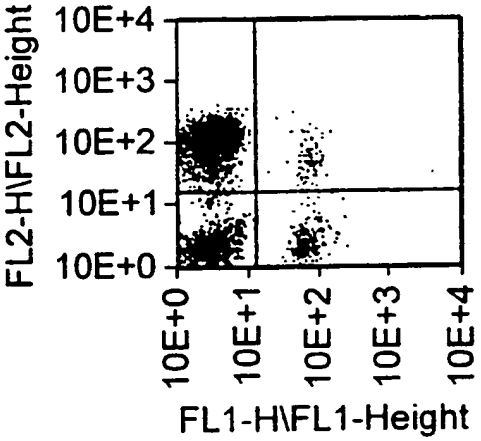

Fig. 24o

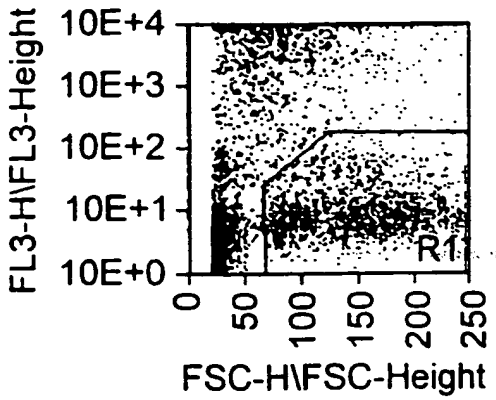

Fig. 25a

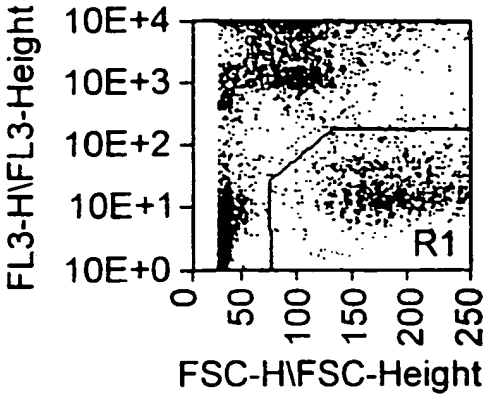

Fig. 25b

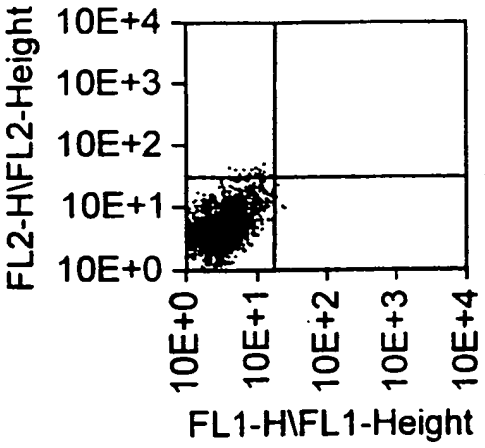

Fig. 25c

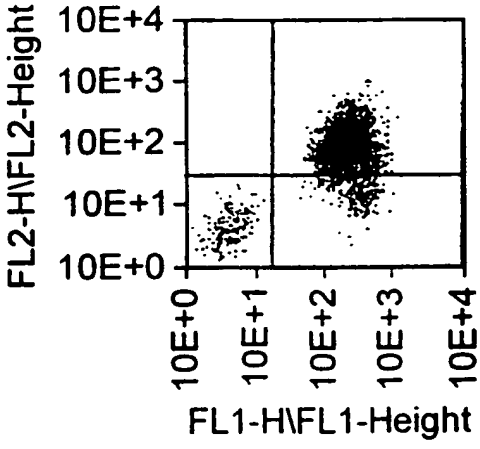

Fig. 25d

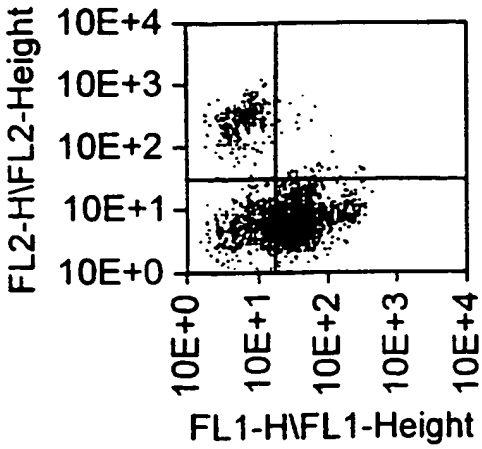

Fig. 25e

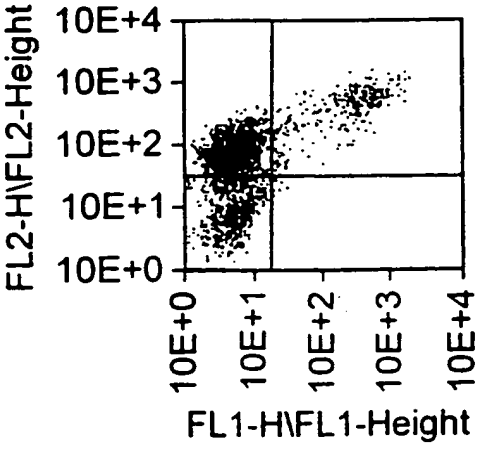

Fig. 25f

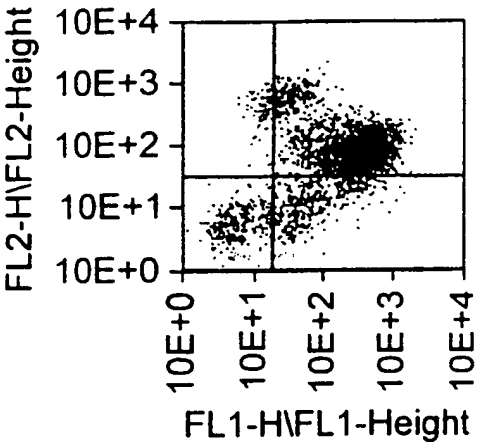

Fig. 25g

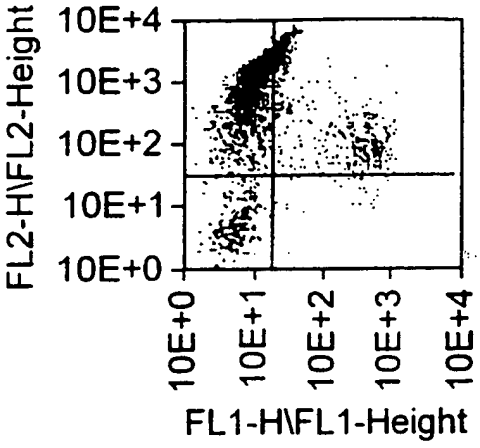

Fig. 25h

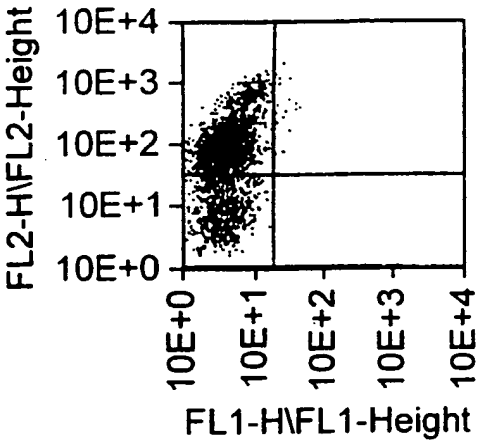

Fig. 25i

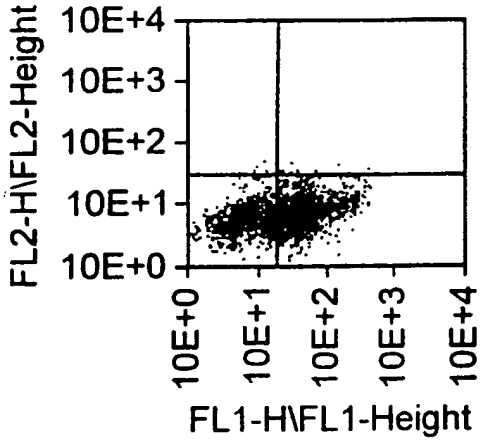

Fig. 25j

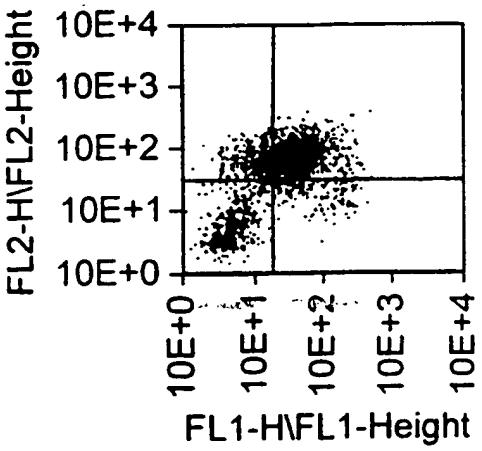

Fig. 25k

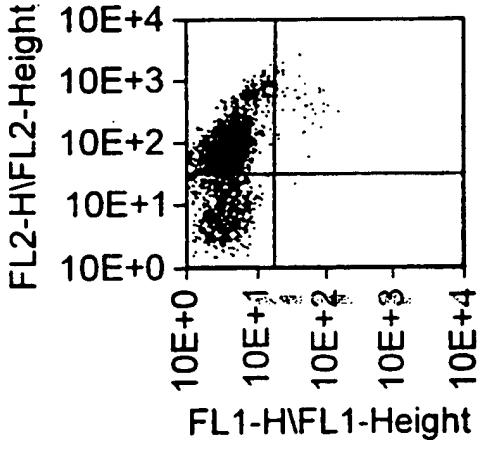

Fig. 25l

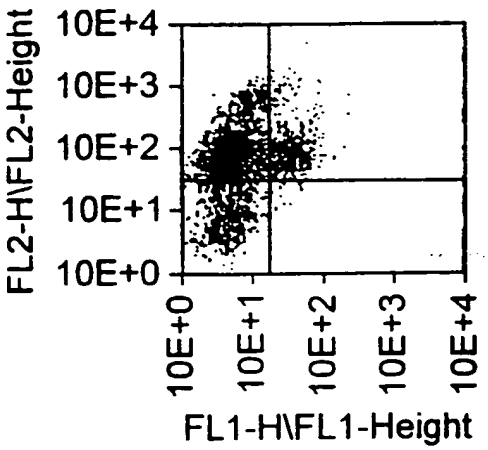

Fig. 25m

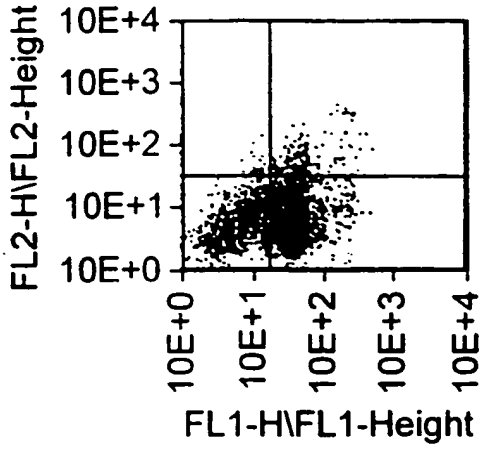

Fig. 25n

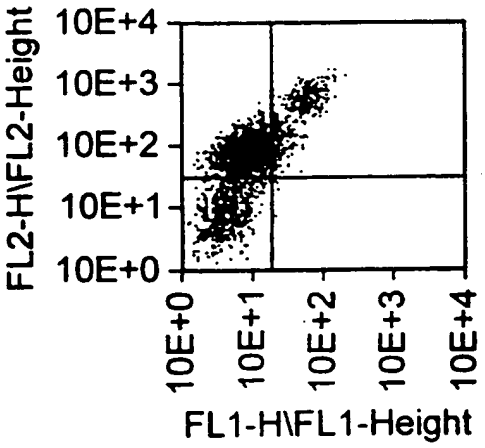

Fig. 25o

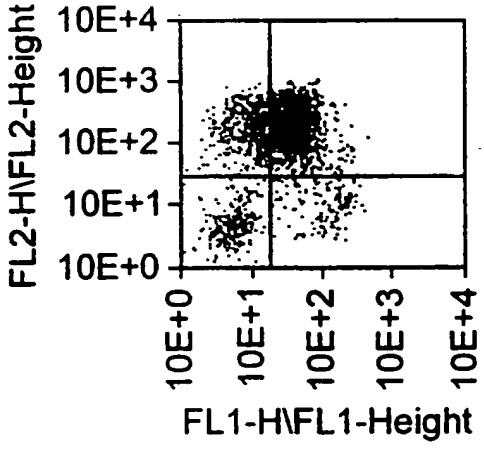

Fig. 25p

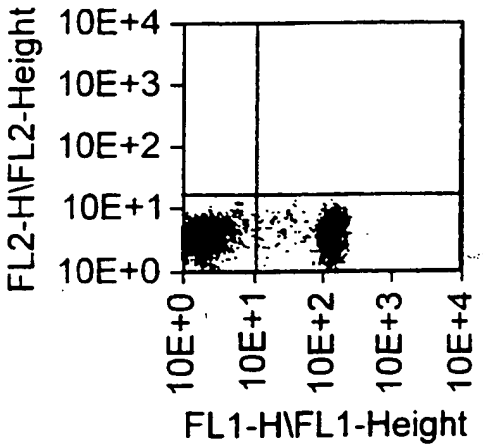

Fig. 26a

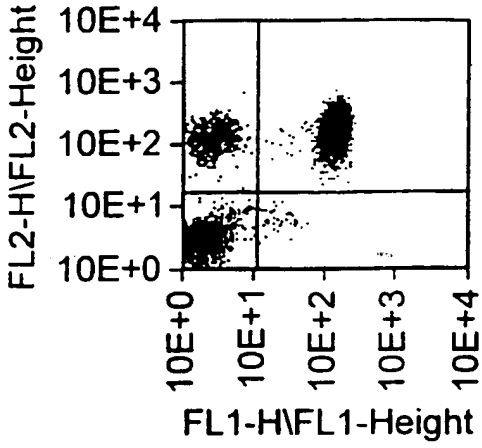

Fig. 26b

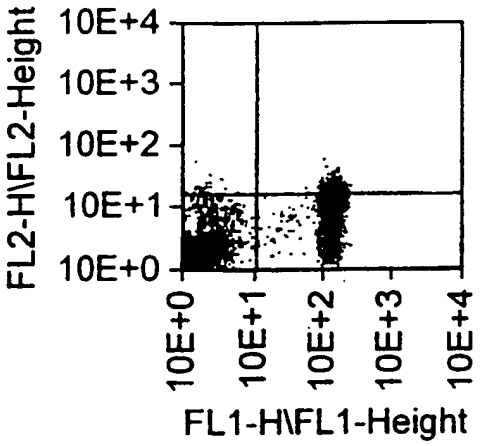

Fig. 26c

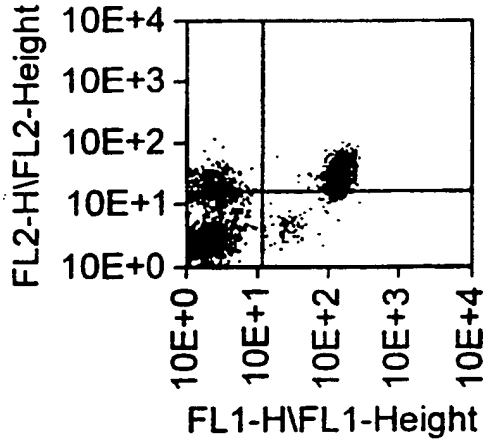

Fig. 26d

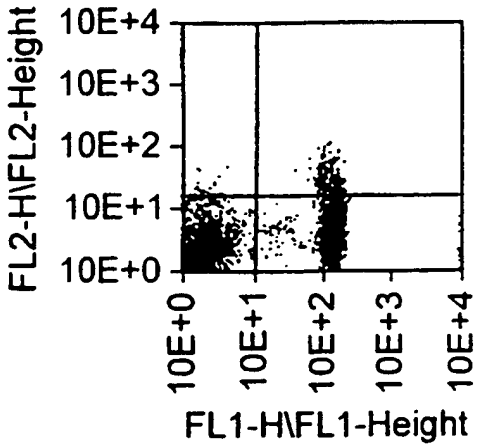

Fig. 26e

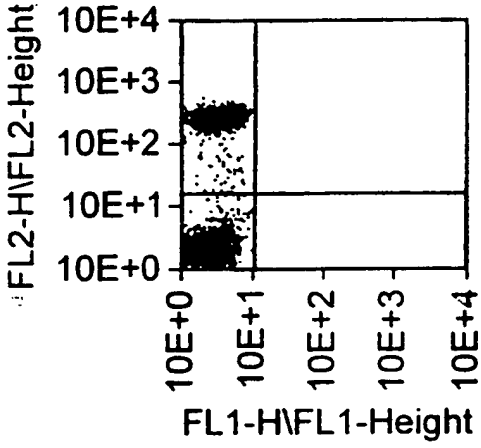

Fig. 26f

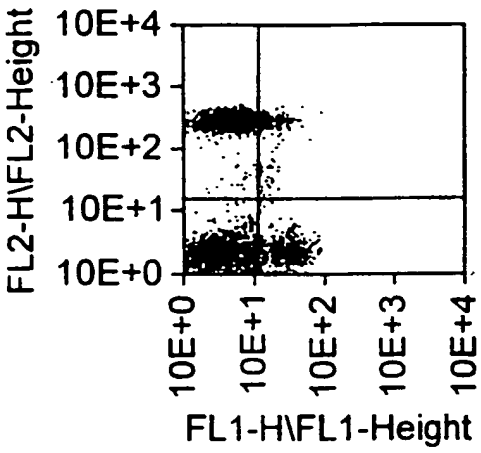

Fig. 26g

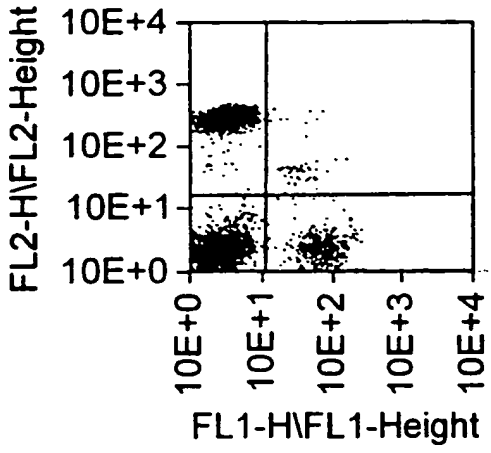

Fig. 26h

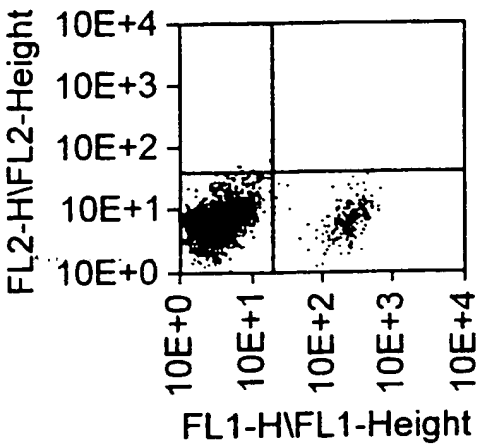

Fig. 27a

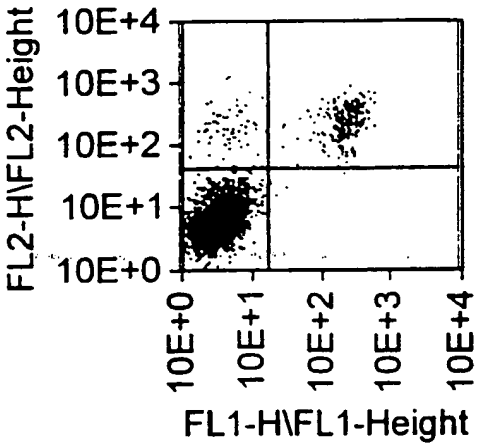

Fig. 27b

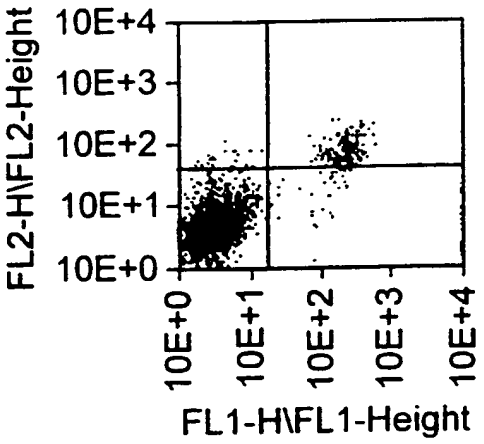

Fig. 27c

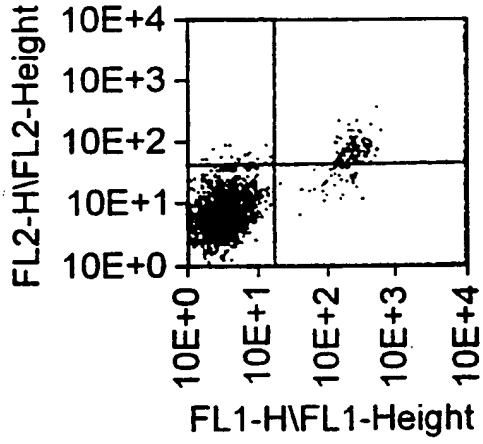

Fig. 27d

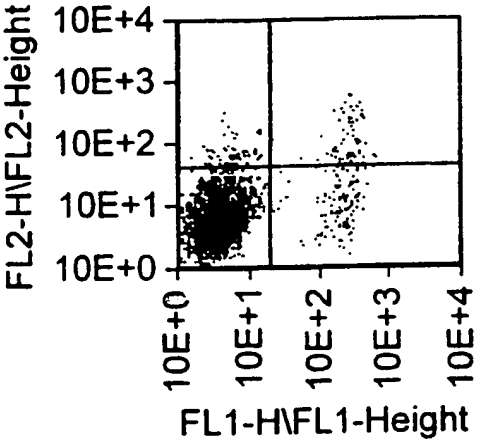

Fig. 27e

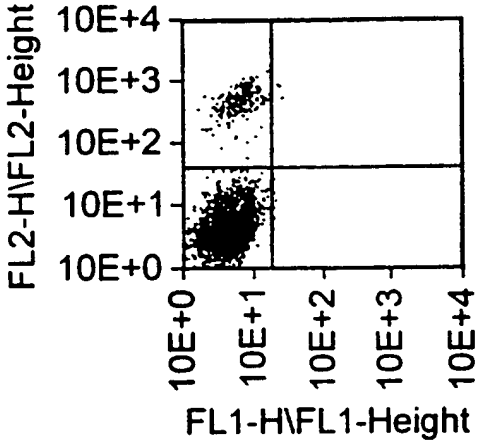

Fig. 27f

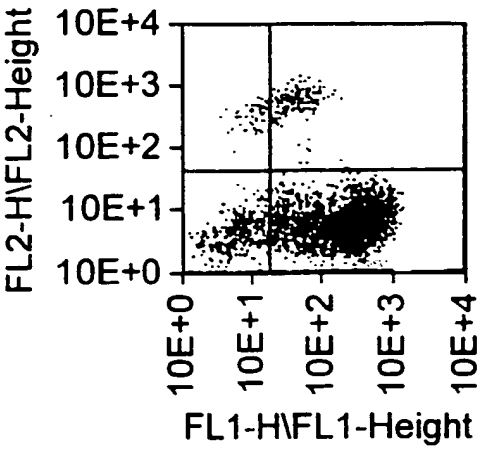

Fig. 27g

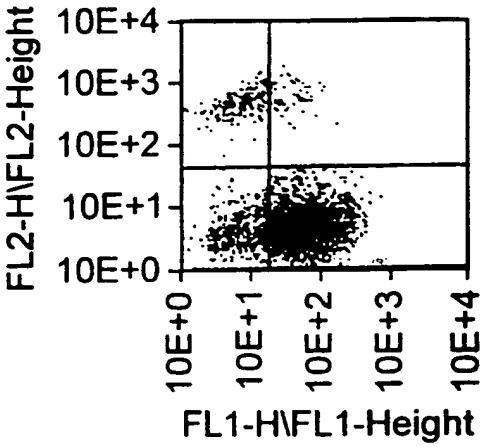

Fig. 27h

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/EP 99/03605

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01K67/027 C12N5/28 C12P21/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 322 240 A (UNIV LELAND STANFORD JUNIOR) 28 June 1989 (1989-06-28) page 2, line 1-26 page 2, line 45 - page 3, line 58 page 4, line 36-44 page 8, line 35-46 claims 23,26	1-18
Y	US 5 663 481 A (GALLINGER STEVEN ET AL) 2 September 1997 (1997-09-02) cited in the application column 4, line 64 - column 5, line 64 column 14, line 13 - column 17, line 14 example 1	1-18

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 1999	02/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

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INTERNATIONAL SEARCH REPORT

Ir ational Application No
PCT/EP 99/03605

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 05796 A (SCRIPPS RESEARCH INST) 1 April 1993 (1993-04-01) page 3, line 5-32 page 6, line 23-27 page 21, line 12-24 page 38, line 27 - page 45, line 34	1-18
A	GREINER D L ET AL: "Improved engraftment of human spleen cells in NOD/LtSz-scid/scid mice as compared with C.B-17-scid/scid mice." AMERICAN JOURNAL OF PATHOLOGY, (1995 APR) 146 (4) 888-902. , XP002112636 cited in the application abstract page 889, left-hand column, paragraph 4 - right-hand column, paragraph 2 page 890, left-hand column, line 18-21 page 895, right-hand column, paragraph 3 - page 898, left-hand column, paragraph 1 page 900, left-hand column, paragraph 3 figure 11	1-18
A	WO 96 39810 A (SANDOZ LTD ;SYSTEMIX INC (US); SANDOZ AG (AT); SANDOZ AG (DE)) 19 December 1996 (1996-12-19) page 3, paragraph 2 page 4, paragraph 2 page 5, paragraph 2 example 1	1-18

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/EP 99/03605

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 15,16 (partially)
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 99 /03605

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 15,16 (partially) are directed to a diagnostic method practised on the human/animal body or to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 15,16 (partially)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy and Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/EP 99/03605

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0322240 A	28-06-1989	AT 119197 T	15-03-1995
		AU 2754888 A	29-06-1989
		DE 3853201 D	06-04-1995
		DE 3853201 T	14-09-1995
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		ES 2071620 T	01-07-1995
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